TURUN YLIOPISTON JULKAISUJA
ANNALES UNIVERSITATIS TURKUENSIS

SARJA - SER. D OSA - TOM. 894

MEDICA - ODONTOLOGICA

INACTIVE ENDOSIALIDASE-BASED DETECTION OF BACTERIAL AND ONCOFETAL POLYSIALIC ACID

by

Anne Jokilammi

TURUN YLIOPISTO
UNIVERSITY OF TURKU
Turku 2010

From the Department of Medical Biochemistry and Genetics, University of Turku
and the Glycoscience Graduate School, Finland.

Supervised by

Professor Jukka Finne, MD, PhD
Department of Medical Biocemistry and Genetics,
University of Turku
Turku, Finland

Reviewed by

Docent Anne Leppänen, PhD
Department of Biological and Environmental Sciences,
University of Helsinki,
Helsinki, Finland

and

Professor Mikael Skurnik, PhD
Haartman Institute,
Department of Bacteriology and Immunology,
University of Helsinki,
Helsinki, Finland

Opponent

Professor Markku Tammi, MD, PhD
Institute of Biomedicine, Anatomy,
University of Eastern Finland,
Kuopio, Finland

ISBN 978-951-29-4211-4 (PRINT)
ISBN 978-951-29-4212-1 (PDF)
ISSN 0355-9483
Painosalama Oy -Turku, Finland 2010

To my parents

Anne Jokilammi

Inactive endosialidase-based detection of bacterial and oncofetal polysialic acid

Department of Medical Biochemistry and Genetics,
University of Turku, Turku, Finland
and the Glycoscience Graduate School, Finland
Annales Universitatis Turkuensis
Painosalama Oy, Turku, Finland 2010

ABSTRACT

Polysialic acid is a carbohydrate polymer which consist of N-acetylneuraminic acid units joined by α2,8-linkages. It is developmentally regulated and has an important role during normal neuronal development. In adults, it participates in complex neurological processes, such as memory, neural plasticity, tumor cell growth and metastasis. Polysialic acid also constitutes the capsule of some meningitis and sepsis-causing bacteria, such as *Escherichia coli* K1, group B meningococci, *Mannheimia haemolytica A2* and *Moraxella nonliquefaciens*. Polysialic acid is poorly immunogenic; therefore high affinity antibodies against it are difficult to prepare, thus specific and fast detection methods are needed.

Endosialidase is an enzyme derived from the *E. coli* K1 bacteriophage, which specifically recognizes and degrades polysialic acid. In this study, a novel detection method for polysialic acid was developed based on a fusion protein of inactive endosialidase and the green fluorescent protein. It utilizes the ability of the mutant, inactive endosialidase to bind but not cleave polysialic acid. Sequencing of the endosialidase gene revealed that amino acid substitutions near the active site of the enzyme differentiate the active and inactive forms of the enzyme.

The fusion protein was applied for the detection of polysialic acid in bacteria and neuroblastoma. The results indicate that the fusion protein is a fast, sensitive and specific reagent for the detection of polysialic acid. The use of an inactive enzyme as a specific molecular tool for the detection of its substrate represents an approach which could potentially find wide applicability in the specific detection of diverse macromolecules.

Key words: polysialic acid, endosialidase, inactivated enzyme, bacteriophage

Anne Jokilammi

Inaktiivisen endosialidaasin käyttöön perustuva menetelmä bakteriaalisen ja onkofetaalisen polysialohapon osoittamiseksi

Lääketieteellinen biokemia ja genetiikka, Turun yliopisto, sekä valtakunnallinen Glykotieteiden tutkijakoulu
Annales Universitatis Turkuensis
Painosalama Oy, Turku 2010

TIIVISTELMÄ

Polysialohappo on N-asetyylineuramiinihappoyksiköistä muodostuva hiilihydraattipolymeeri, jonka yksiköt ovat liittyneet toisiinsa α2,8-sidoksin. Polysialohapolla on merkittävä rooli normaalin neuronaalisen kehityksen aikana ja yksilön kehitysvaiheet säätelevät sen esiintymistä. Aikuisilla se on osallisena monimutkaisissa neurologisissa prosesseissa kuten muistamisessa, neuronaalisessa muovautuvuudessa (plastisuudessa) sekä syöpäsolujen kasvussa ja metastasoinnissa. Polysialohappo muodostaa myös eräiden sepsistä ja meningiittiä aiheuttavien bakteerien pinnalla olevan kapselin. Tällaisia bakteereita ovat esimerkiksi *E. coli* K1, ryhmän B meningokokki, *Mannheimia haemolytica A2* sekä *Moraxella nonliquefaciens*. Polysialohapon spesifiselle ja nopealle analysointimenetelmälle on tarvetta, koska sen heikon immunogeenisyyden takia tehokkaita vasta-aineita on ollut vaikea valmistaa.

Endosialidaasi on *Escherichia coli* K1:n bakteriofagista peräisin oleva entsyymi, joka spesifisesti tunnistaa ja pilkkoo polysialohappoa. Tässä työssä kehitettiin polysialohapon osoittamiseksi menetelmä, joka perustuu inaktiivisen endosialidaasi-entsyymin käyttöön. Kehitetyssä fuusioproteiinissa inaktiivinen endosialidaasi on liitetty vihreään fluoresoivaan proteiiniin. Menetelmä hyödyntää inaktiivisen mutanttiendosialidaasin kykyä sitoutua polysialohappoon sitä pilkkomatta. Endosialidaasigeenin sekvensointi paljasti, että lähellä entsyymin aktiivista keskusta sijaitsevan kahden aminohapon muutos erottaa entsyymin aktiivisen ja inaktiivisen muodon toisistaan.

Fuusioproteiinia käytettiin polysialohapon osoittamiseen bakteereissa ja neuroblastoomassa. Inaktiivisen entsyymin käyttö spesifisenä reagenssina, substraattimolekyylinsä merkkiaineena, edustaa lähestymistapaa, jota voitaisiin mahdollisesti käyttää laajemminkin erilaisten makromolekyylien spesifiseen osoittamiseen.

Avainsanat: polysialohappo, endosialidaasi, inaktivoitu entsyymi, bakteriofagi

TABLE OF CONTENTS

ABBREVIATIONS

BHK-21	A baby hamster kidney cell line
cryoEM	Cryo-electron microscopy
CSF	Cerebrospinal fluid
CTD	C-terminal domain
CUS-3	*E. coli* K1 prophage
DMB/HPLC-FD	1,2-diamino-4,5-methylenedioxybenzene / high-performance liquid chromatography with fluorescence detection
DP	Degree of polymerization
EMBL	European Molecular Biology Laboratory
endoN	Endosialidase, endo-N-acylneuraminidase
endoNF	Endosialidase F isolated from the phage K1F
GFP	Green fluorescent protein
HPAEC-PAD	High-performance-anion-echange cromatography with pulsed amperometric detection
HPLC	High-performance liquid chromatography
KDN	Deaminated neuraminic acid
Men B	Group B meningococcus
MALDI-TOF-MS	Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
NCAM	Neural cell adhesion molecule
Neu5Ac	N-acetylneuraminic acid
Neu5Gc	N-glycolylneuraminic acid
NMR	Nuclear magnetic resonance
PCR	Polymerase chain reaction
PDB	Protein Data Bank
PEG	Polyethyleneglycol
S-D	Shine-Dalgarno
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electroforesis
SH-SY5Y	A human neuroblastoma cell line
TMFU	Trifluoromethylumbelliferyl

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by the Roman numerals (I-IV):

I Jokilammi, A., Ollikka, P., Korja, M., Jakobsson, E., Loimaranta, V., Haataja, S., Hirvonen, H. and Finne, J. (2004)
Construction of antibody mimics from non-catalytic enzyme – detection of polysialic acid.
J. Immunol. Meth. 295, 149-160.

II Jakobsson, E.[1], Jokilammi, A.[1], Aalto, J., Ollikka, P., Lehtonen, J., Hirvonen, H. and Finne, J. (2007)
Identification of the amino acid residues at the active site of endosialidase that dissociate the polysialic acid binding and cleaving activities in *Escherichia coli* K1 bacteriophages.
Biochem. J. 405, 465-472.
[1]These authors contributed equally to this work

III Zelmer, A., Bowen, M., Jokilammi, A., Finne, J., Luzio, P. and Taylor, P.W. (2008)
Differential expression of the polysialyl capsule during blood-to-brain transit of neuropathogenic *Escherichia coli* K1.
Microbiology 154, 2522-2532.

IV Jokilammi, A., Korja, M., Jakobsson, E. and Finne, J. (2007)
Generation of lectins from enzymes: Use of inactive endosialidase for polysialic acid detection.
Lectins: Analytical Technologies, Nilsson, C.L., ed., Elsevier, 385-395.

1 INTRODUCTION

Polysialic acid is a carbohydrate polymer consisting of N-acetylneuraminic acid units joined together by α2,8-linkages. It is found both in bacterial and eukaryotic cells. The degree of polymerisation of polysialic acid chains varies. In human neuroblastoma, the average chain length is 50-60 sialic acid units or less. However, a subpopulation of chains between 150 and 400 residues has been observed for neuroblastoma cells transfected with polysialyltransferase (Nakata *et al.*, 2005). In *Escherichia coli* K1, the chain length is over 200 sialic acid units (Rohr *et al.*, 1980).

Polysialic acid has been ascribed important roles in neural development, cell differentiation and plasticity. It is also expressed in neural malignancies and has been reported to favor tumor cell growth and metastasis (Tanaka *et al.*, 2000; Seidenfaden *et al.*, 2003; Cheung *et al.*, 2006). In prokaryotes polysialic acid is found as a capsular polysaccharide and a virulence factor of some neurotrophic bacterial pathogens. Many methods have been developed for the detection of polysialic acid: these methods, however, are often laborious and time consuming. Therefore, faster and more specific methods are needed.

Bacteriophages, bacterial viruses, are widely distributed in nature, and particularly abundant in sea water. These organisms could potentially serve as a wide reservoir for new reagents for targeting macromolecules. *E. coli* K1-specific bacteriophages have enzyme endosialidase as a tailspike. These phages use endosialidase for the recognition of the bacteria, binding to them and degrading their protective polysialic acid-capsule. Previously, phage mutants have been reported that have lost their polysialic acid cleaving activity while retaining their binding activity for polysialic acid (Pelkonen *et al.*, 1992).

The aim of this study was to determine whether the inactive endosialidase can be used for the development of a new detection method for polysialic acid. Inactive endosialidase was therefore fused into a reporter molecule, a green fluorescent protein, and the fusion protein was explored as a novel detection method for the polysialic acid in an infection model and in the staining of neuroblastoma sections.

In this work, the difference between the mutant and native forms of endosialidase was characterized by sequencing and back mutations to identify the molecular basis for the inactivation. It was found that inactive and active forms of endosialidase differ only by two amino acids, which in a homology-based model were observed to be located near the active site of the enzyme.

2 REVIEW OF THE LITERATURE

2.1 Polysialic acid

Polysialic acids are long and linear homopolymers. Their structure consist of *N*-acetylneuraminic acid (Neu5Ac), *N*-glycolylneuraminic acid (Neu5Gc) or deaminated neuraminic acid (KDN) residues (Figure 1), which are joined usually by α2,8, α2,9 or alternating α2,8/α2,9 ketosidic linkages. The predominant form of polysialic acid in mammalian tissues is the polymer consisting of N-acetylneuraminic acid units linked to each other by α2,8 linkages (Figure 2). Most commonly, polysialic acid is used as a synonym for the α2,8-linked polymer, and this review therefore focuses on this type of polysialic acid.

In eukaryotics, polysialic acid is mainly found as a part of the neural cell-adhesion molecule, NCAM, the cell-adhesion properties of which it modulates (Finne 1982; Fujimoto *et al.*, 2001; Johnson *et al.*, 2005a; Johnson *et al.*, 2005b; Rutishauser 2008). NCAM is a cell surface molecule which is expressed in the nervous system and in various cell types, such as natural killer cells, cardiomyocytes, and neuroendocrine cells (Kibbelaar *et al.*, 1991; Mechtersheimer *et al.*, 1991). NCAM is the main carrier of polysialic acid in eukaryotes (Finne *et al.*, 1983). As it is a negatively charged, hydrophilic polymer, polysialic acid has a large space-requirement which causes steric hindrances in cell-cell adhesion (Yang *et al.*, 1994; Johnson *et al.*, 2005b). The polysialic acid in NCAM is synthesized by two polysialyltransferases, ST8Sia II (STX) and ST8Sia IV (PST) (Tsuji 1996; Mühlenhoff *et al.*, 1998; Ong *et al.*, 1998). These two enzymes act on NCAM slightly differently, but cooperatively, and catalyse together the synthesis of a larger amount polysialic acid than separately (Angata *et al.*, 2003). Polysialic acid has also been suggested to occur in the α-subunit of the voltage-gated sodium channel in the mammalian brain and muscle (James *et al.*, 1987).

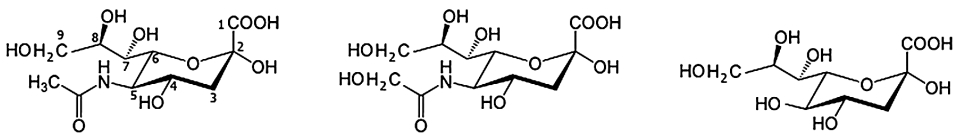

Figure 1. The structure of *N*-acetylneuraminic acid (Neu5Ac), *N*-glycolylneuraminic acid (Neu5Gc) and deaminated neuraminic acid (KDN), the building units of polysialic acid chains. These sialic acids are neuraminic acid derivatives, where at position C-5 Neu5Ac has an N-acetyl group, Neu5Gc hydroxylated an N-acetyl group and KDN a hydroxyl group.

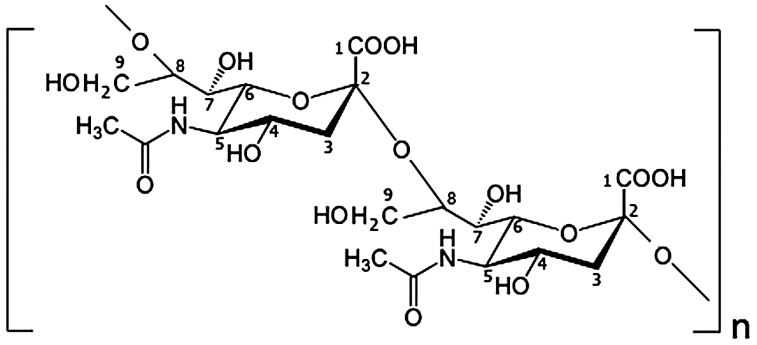

Figure 2. The structure of α2,8 polysialic acid consisting of N-acetylneuraminic acid units.

In prokaryotes, polysialic acid forms the protecting capsule polysaccharide of some pathogenic bacteria. This polysialic acid is structurally identical to that found in eukaryotes (Finne *et al.*, 1983). Capsule production has been often associated with virulence and the invasion of bacteria (Taylor *et al.*, 2005).

2.1.1 Polysialic acid in mammals

Eukaryotic polysialic acid was first detected in rainbow trout eggs (Inoue *et al.*, 1978), but its discovery in the mammalian brain (Finne 1982) inspired intensive research in neuroscience. The expression of polysialic acid is developmentally regulated (Finne *et al.*, 1987; Kiss *et al.*, 1997; Ong *et al.*, 1998), and it is the most strongly expressed during embryogenesis, where polysialic acid is reported to promote plasticity of the cell-cell interactions during neuronal development (Rutishauser *et al.*, 1996; Brusés *et al.*, 2001; Rutishauser 2008). In the adult brain, its expression is reduced to restricted areas related to neural regeneration (Cremer *et al.*, 1994; Bonfanti 2006) as well as to memory (Doyle *et al.*, 1992) and learning, especially spatial learning (Cremer *et al.*, 1994). High levels of polysialic acid are detected only in those regions of the nervous system that retain neurogenic capacity or are plasticity-associated (Seki *et al.*, 1993; Muller *et al.*, 1996; Gascon *et al.*, 2008). Polysialic acid is involved in the modulation of activity in the hypothalamic region, where the circadian clock is situated. A large proportion of the research on polysialic acid is focused on neuronal development, but polysialic acid is also expressed during the development and regeneration of some other tissues, such as the kidney (Roth *et al.*, 1987), heart (Lackie *et al.*, 1991) and muscle (Dubois *et al.*, 1994).

The polysialylated form of NCAM has been widely studied in the developing nervous system. Previous studies have shown that polysialic acid has a central role in many neuronal functions, such as cell migration (Murakami *et al.*, 2000; Zhang *et al.*, 2004), cytokine response (Vutskits *et al.*, 2001; Zhang *et al.*, 2004), and cell contact-dependent differentiation (Petridis *et al.*, 2004). These

functions have analogous functions in the immune system. Leukocytes migrate throughout the body guided by specialized chemokines, affecting both homeostatic and inflammatory functions that are often dictated by cytokine and cell contact-dependent signals. Polysialic acid expression on NCAM in human NK cells, as well as mouse hematopoietic progenitors and myeloid cells suggest a role for polysialic acid also in the immune system (Drake *et al.*, 2008).

2.1.2 Polysialic acid in neurological diseases

Altered levels of polysialic acid can be detected in various neurological diseases, such as Alzheimer's disease, schizophrenia and epilepsy. In patients with Alzheimer´s disease, the expression of polysialylated NCAM is increased in the brain as a response to degeneration. The highly charged polysialic acid epitopes of NCAM may alter the adhesive properties of the remaining neuronal structures, and this way provides an opportunity for reinnervation (Mikkonen *et al.*, 1999). Similar results have also been described in epilepsy (Mikkonen *et al.*, 1998). In schizophrenic hippocampi, a decrease of polysialic acid is supposed to reflect the altered plasticity, and the reduction of polysialic acid could have an important role in the pathophysiology of schizophrenia (Barbeau *et al.*, 1995). Repeated, chronic stress increases the expression of polysialylated NCAM, and prolonged stress can cause irreversible damage to recently born cells in the hippocampal area (Pham *et al.*, 2003).

2.1.3 Expression of polysialic acid in tumors

Although polysialic acid is virtually absent in the majority of adult tissues, it has been found re-expressed in some malignant tumors and cell lines (Table 1). In these tumors, polysialic acid has been associated with invasion and a metastatic tendency (Hildebrandt *et al.*, 1998; Tanaka *et al.*, 2000; Seidenfaden *et al.*, 2003; Suzuki *et al.*, 2005; Cheung *et al.*, 2006). It also facilitates the detachment of cancer cells from the primary tumor (Scheidegger *et al.*, 1994). Earlier studies have shown that the polysialylated form of NCAM can be used as a diagnostic and possibly a prognostic marker of several tumors. Some neuroblastomas express polysialylated NCAM (Livingston *et al.*, 1988; Glüer *et al.*, 1998a), and recently, polysialic acid has been suggested as a molecular marker in the risk stratification of neuroblastoma patients (Korja *et al.*, 2009).

Table 1. Expression of polysialic acid in human tumors and cell lines. References for the cell lines are marked by an asterisk.

Tumor/cell line	References
Neuroblastoma	Lipinski *et al.*, 1987*
	Glick *et al.*, 1991
	Glüer *et al.*, 1998a
Wilms tumor	Roth *et al.*, 1988
Medulloblastoma	Figarella-Branger *et al.*, 1996
Small cell lung cancer	Kibbelaar *et al.*, 1989
	Moolenaar *et al.*, 1990*
Non-small cell lung cancer	Tanaka *et al.*, 2000
Multiple myeloma	Van Camp *et al.*, 1990
Rhabdomyosarcoma	Glüer *et al.*, 1998b
Natural killer cell derived-lymphoma	Kern *et al.*, 1992
Astrocytoma	Petridis *et al.*, 2009
Pancreatic tumor cells	Schreiber *et al.*, 2008*
Breast cancer cells	Martersteck *et al.*, 1996*

2.1.4 Bacterial polysialic acid

Capsular polysaccharides are important virulence factors of pathogenic bacteria causing severe invasive infections, such as septicemia, meningitis, pneumonia, osteomyelitis, septic arthritis and pyelonephritis (Cross *et al.*, 1990; Moxon *et al.*, 1990; Taylor *et al.*, 2005). The capsule is formed by a thick (400 nm or more) layer protecting the bacterium from the threats of environment and host immune defence. The capsule protects the bacterial surface structures from complement-mediated lysis, which is an important step in the beginning of infection. On the other hand, the capsule is also a good target for the attachment of bacteriophages (Stirm *et al.*, 1971).

Sialic acid polymers were discovered on the surface of *E. coli* as early as the end of 1950s (Barry *et al.*, 1957; Barry 1958). Later, polysialic acid has been found to constitute the capsule of *E. coli* K1 (Robbins *et al.*, 1974) and some other meningitis and septicemia-causing bacteria, such as *Neisseria meningitidis* group B (Liu *et al.*, 1971; Bhattacharjee *et al.*, 1976), *Mannheimia (Pasteurella) haemolytica* A2 (Jennings *et al.*, 1984) and *Moraxella nonliquefaciens* (Bøvre *et al.*, 1983). The capsule structure is identical to mammalian polysialic acid (Finne *et al.*, 1983). A recent study that compared polysialic acid structures *in vivo* and *in vitro* using nuclear magnetic resonance (NMR) suggests that environmental factors, such as pH, could have a significant influence on the structural and dynamic properties of this polymer (Azurmendi *et al.*, 2007). Additional structures in polysialic acid chain linkages, such as lactones, could rigidify the polymer structure. At neutral pH, α2,8-linkages form a flexible linear polymer, but at low pH the number of lactones

increases (Figure 3). Lactones have been reported to reduce the antigenicity of polysialic acid (Lifely *et al.*, 1981).

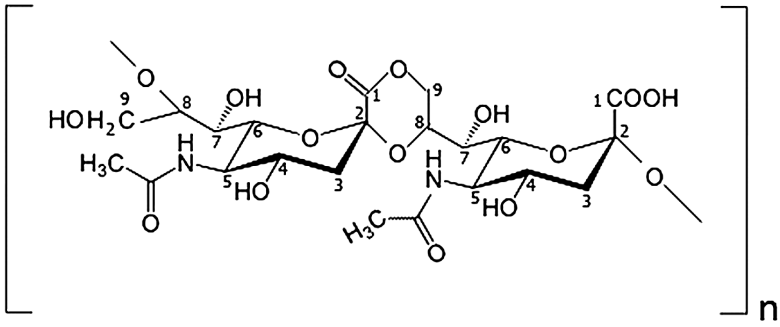

Figure 3. The structure of α2,8 polysialic acid with a lactone ring. A lactone is formed when a carboxylic acid group condensates with the hydroxyl group in carbon nine of another residue, and forms a six-membered ring.

E. coli K1 is a predominant pathogen of neonatal sepsis and meningitis (Robbins *et al.*, 1974; Sarff *et al.*, 1975) and is associated with high rates of mortality, morbidity and severe neurologic sequelae (Sáez-Llorens *et al.*, 2003; Kaper *et al.*, 2004). The K1 capsule polysaccharide is composed of up to 200 α2,8-linked sialic acid units and is poorly immunogenic due to the structural identity to host polysialic acid (Mühlenhoff *et al.*, 1998). A eukaryotic polysialic acid-mimicking capsule hence allows the pathogen to escape the influence of the immune system (Cross *et al.*, 1986). The capsular polysaccharide is a major virulence factor of *E. coli* K1 and important for its serum resistance (Leying *et al.*, 1990). Some bacteria, such as *E. coli*, *M. haemolytica* and *N. meningitidis* appear to need the polysialic acid capsule also for crossing the blood brain barrier to cause neuroinvasive disease (Kim *et al.*, 2003), but the molecular mechanisms involved have so far not been characterized.

2.1.5 Examples of practical applications based on polysialic acid

Based on its ability to regulate plasticity (Rutishauser 2008), polysialic acid has been applied therapeutically in mouse models to augment tissue repair in the central nervous system. Induction of polysialic acid expression at the site of damage has been studied in the regeneration of axons and in recruitment of endogenous progenitors to brain injury (El Maarouf *et al.*, 2006). In such experimental systems, polysialic acid can create favorable conditions for the repair of a damaged tissue. It helps axons to regrow beyond the injury site, and it may increase the number of progenitor cells at the site of injury and provide an environment where the cells start to differentiate (El Maarouf *et al.*, 2006).

Restorative medicine has a need for new materials to replace injured organs and tissues with reconstructed grafts. Among various polymers studied for this

purpose (Langer *et al.*, 1993), chemically modified polysialic acid has been suggested as a scaffold material for tissue engineering (Berski *et al.*, 2008). It has been shown that a polysialic acid-based hydrogel in combination with cell adhesion molecules and growth factor-expressing cells could be promising in therapeutic strategies (Haile *et al.*, 2008). As a scaffold material polysialic acid-based hydrogel has many advantages, such as easy and specific removal with a bacteriophage-derived endosialidase (Hallenbeck *et al.*, 1987a). Its degradation products are not toxic, which is an important feature (Haile *et al.*, 2007). Degradability may speed up recovery after surgery because ablation of the hydrogel is not needed.

Peptides and proteins are promising therapeutic agents, but many of them are difficult to use with patients. They can be unstable in the body, have too rapid a clearance rate, and too early uptake by tissues, or their immunogenicity or antigenicity can cause problems (Harris *et al.*, 2003). These problems have been approached by pegylation, where polyethylene glycol (PEG) chains are attached to peptide or protein drugs. This reduces the renal clearance by increasing the molecular mass and protects the molecules from proteolytic enzymes. However, PEG is nonbiodegrable, and its accumulation in tissues may cause toxic effects. Polysialic acid has been suggested as an alternative conjugate to PEG to modulate *in vivo* pharmacokinetics of peptide and protein drugs, and to improve their stability (Gregoriadis *et al.*, 2005). Such conjugates have been prepared with asparaginase (Fernandes *et al.*, 1997) and insulin (Jain *et al.*, 2003) for the treatment of leukemia and diabetes, respectively. In both cases the serum half-life of the conjugate was increased, which means that the potential drug has more time to reach its target and act therapeutically before clearance. Polysialylation of a Fab antibody fragment specific to germ cell carcinoma has also been investigated, and the results show that polysialylation extends the half-life of the antibody fragments, and improves their uptake by the tumor (Constantinou *et al.*, 2008; Constantinou *et al.*, 2009).

2.2 Endosialidase

The endosialidase, or endo-*N*-acetylneuraminidase (endoN), (EC 3.2.1.129, a glycosyl hydrolase) is a tailspike enzyme of bacteriophages specific for *E. coli* K1, which specifically recognizes and degrades polysialic acid. The genes encoding it have been cloned from some of the phages (Gerardy-Schahn *et al.*, 1995; Long *et al.*, 1995; Scholl *et al.*, 2001). In contrast to exosialidases, which cleave terminal α-ketosidically linked sialic acid residues, endosialidases require oligo- or polymers of α2,8-ketosidically linked sialic acids (Finne *et al.*, 1985; Hallenbeck *et al.*, 1987a; Pelkonen *et al.*, 1989). Endosialidases are only found in K1-specific phages, and similar polysialic acid-degrading enzymes have not been discovered in other organisms so far.

2.2.1 *E. coli* K1 bacteriophages

According to the definition, bacteriophages are viruses of bacteria. They are highly abundant and versatile, and act in many natural ecosystems. Phages can be divided into lytic and temperate types, based on their life cycles. The lytic phage infection causes lysis of the bacterial cell and release of a new phage progeny. The life cycle of the temperate phages can be lytic or lysogenic, and most lysogenic phages acquire a prophage state, where the genome of the phage is replicated synchronically with the host bacterial chromosome. In the process of infection the phage attaches specifically to the surface of the host bacterial cell with its primary adhesin, which in tailed phages is often the tail fiber or spike.

Tailed phages (*Caudovirales*), the largest group of bacteriophages, have a common origin and tail spikes or fiber proteins for efficient host interactions (Ackermann 1998; Ackermann 2003). During viral infection these adhesins mediate specific recognition and attachment to the bacterial surface. The tailspike or fiber proteins are often enzymatically active since a variety of pathogenic bacteria are protected by a thick capsule of lipopolysaccharides or polysaccharides. Phages that infect polysaccharide encapsulated bacteria need endoglycosidases or lyases to reach the bacterial membrane (Stirm *et al.*, 1971). Many tailspikes and fibers are built up by homotrimers that contain structural subunits, such as coiled-coil, triple β-helix, or triple β-spiral folds. These structures help protein complexes to remain stable in hard environments, for example in the presence of sodium dodecyl sulfate (SDS) (Weigele *et al.*, 2003).

E. coli K1 phages are lytic bacteriophages specific to *E. coli* K1 and, morphologically, belong to either *podoviridae* with short non-contractile tails, or *myoviridae*, with a long and contractile tail apparatus. They have been isolated from sewage and can be classified to linear double-stranded DNA viruses (Gross *et al.*, 1977; Kwiatkowski *et al.*, 1982; Smith *et al.*, 1982; Kwiatkowski *et al.*, 1983; Vimr *et al.*, 1984; Scholl *et al.*, 2001). A common feature to all K1 phages is the presence of an endosialidase as a tail spike protein, which selectively degrades α2,8-linked polysialic acid (Finne *et al.*, 1985; Hallenbeck *et al.*, 1987a; Pelkonen *et al.*, 1989). The endosialidase tail spike genes have been sequenced and expressed as proteins from *E. coli* K1 bacteriophage K1E (Gerardy-Schahn *et al.*, 1995; Long *et al.*, 1995), K1F (Petter *et al.*, 1993; Mühlenhoff *et al.*, 2003), K1-5 (Scholl *et al.*, 2004) and K1A (this study). *E. coli* K1 prophage CUS-3 (Deszo *et al.*, 2005), a temperate phage with an endosialidase gene, has also been sequenced and expressed as a protein (Stummeyer *et al.*, 2006).

2.2.2 The structure of the endosialidase

Endosialidases share the same common architecture. They have three linearly organized domains: an N-terminal capsid-binding domain, a central catalytic domain and a C-terminal domain. The N-terminal capsid-binding domain fastens the endosialidase tailspike to the phage particle. The length of the

domain varies between different phages and this domain is not needed for enzymatic activity (Mühlenhoff *et al.*, 2003; Stummeyer *et al.*, 2006). The central catalytic domain is highly conserved and it includes both the polysialic acid binding and the cleaving activity of the endosialidase (Stummeyer *et al.*, 2005). The C-terminal domain is short and functions as an intramolecular chaperone, which is released from the mature enzyme (Mühlenhoff *et al.*, 2003; Schwarzer *et al.*, 2007). The C-terminal domain together with the N-terminal capsid-binding domain flanks the catalytic domain (Horn *et al.*, 2006).

Three-dimensional crystal structures have been solved for many exosialidases, but the first, and thus far only, structure of an endosialidase is that of the conserved catalytic domain (aa 246–911) of endosialidase K1F (Stummeyer *et al.*, 2005). The structure has features common to exosialidases, and on the other hand to tailspike proteins of phages. The functional enzyme is a homotrimer and resembles the shape of a mushroom (Figure 4). The cap structure of the mushroom is formed by three six-bladed β-propellers as is characteristic for sialidases, and each of them has an active site. Additionally, a lectin-like β-barrel domain extends from each β-propeller and contains a polysialic acid binding site. The foot structure is built by the intertwining C-terminal portions and is composed of a triple β-helix that is covered by a short triple β-prism domain. These folds are suggested to contribute to the stability of the protein, and both of them are also found in tailspike proteins of other phages (Weigele *et al.*, 2003). The stalk domain stabilizes the trimer and also participates in substrate binding, because it has a sialic acid binding site (Stummeyer *et al.*, 2005).

The structure of the C-terminal domain (CTD) has not yet been determined. The CTD is highly conserved and is partly homologous with domains of tailspike and fiber proteins of different phages. This supports the observation that the CTD has a role in the folding and assembling of phage proteins (Schwarzer *et al.*, 2007). The CTD acts as an intramolecular chaperone (Mühlenhoff *et al.*, 2003), and is suggested to lower a high-energy barrier of the folding pathway (Schwarzer *et al.*, 2007). During the maturation of endoNF, the CTD is released by proteolytic cleavage at a highly conserved serine residue (Mühlenhoff *et al.*, 2003; Schwarzer *et al.*, 2007). If the serine residue is substituted by alanine, the cleavage is prevented, but the assembly into an active trimer is not. This shows that the complex formation and activity do not require preceding proteolytic processing. However, trimerization of the endosialidase is dependent on an intact CTD in the nascent polypeptide. Truncation or a point mutation in a single histidine residue within the CTD domain prevents the trimerization and causes formation of an inactive and insoluble protein (Gerardy-Schahn *et al.*, 1995; Mühlenhoff *et al.*, 2003).

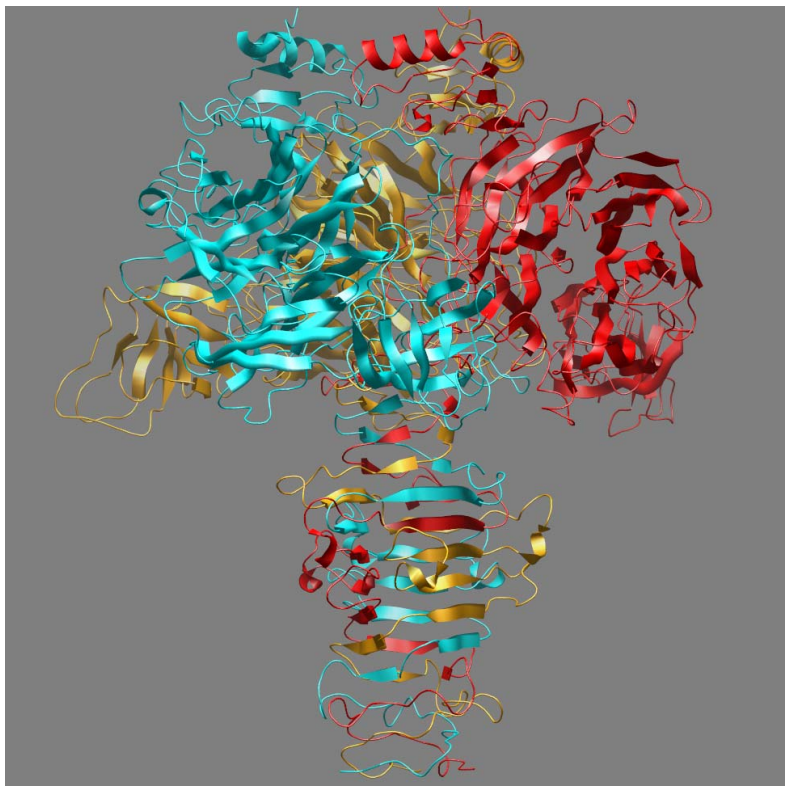

Figure 4. A ribbon diagram of trimeric endosialidase K1F. Monomers are colored in red, gold and cyan blue. The image is made from chains D, E and F of the endosialidase K1F structure (PDB ID: 1V0E, Stummeyer *et al.*, 2005) by using the MALIGN algorithm (Johnson *et al.*, 1993) in the program Bodil (Lehtonen *et al.*, 2004). Courtesy of Jukka Lehtonen.

The CTD is also shown to influence the activity of the endosialidase. The wild-type endoNF has been shown to be a processive enzyme, which means that after initial association the enzyme remains attached to the substrate for several rounds of cleavage (Schwarzer *et al.*, 2009). Mutation in the conserved amino acid residues of the CTD prevents the building of a functional enzyme by destroying the polysialic acid-binding site in the stalk domain (Mühlenhoff *et al.*, 2003). The mutant forms of the endoNF enzyme catalyze the degradation of soluble polysialic acid more effectively, a finding also reported earlier for other processively acting enzymes, such as chitinase (Horn *et al.*, 2006). Proteolytic release of the CTD chaperone stabilizes the endoNF tailspike and has an important role in optimizing the balance between the catalytic and binding functions of the enzyme (Schwarzer *et al.*, 2009).

2.2.3 Evolution of K1-specific phages and their tailspike proteins

All K1 phages studied to date have had endosialidase tailspike proteins. Sequencing of some K1 phages has revealed that not all K1 phages have evolved from a common ancestor, but from different progenitor types, such as T7, SP6 and P22 phages. The K1 phages have developed a new host specificity by replacing the tailspike with an endosialidase. Transmission of the gene has occurred by horizontal gene transfer (Stummeyer *et al.*, 2006).

All endosialidases have a highly conserved catalytic part, a CTD and a variable N-terminal part. The N-terminal domains are required neither for proper folding nor for enzymatic activity. The N-terminal capsid-binding domain of the original spike or fiber protein is presumably retained to ensure proper integration of the new tailspike (Stummeyer *et al.*, 2006).

Two different ways to incorporate new tailspikes have been reported for tailed phages. The original capsid-binding domain of the progenitor phage can be retained, when the capsid-binding and host range determining functions are combined to form a fusion protein. Another possibility is that the new tailspike connects to the tail by an adapter protein. The interaction of adapter protein gp37 is independent of the tailspike, a property that could enable the development of phages with defined host specificity for phage therapy (Stummeyer *et al.*, 2006). Cryo-electron microscopy (CryoEM) constructions show that the tailspikes interact with their adapter proteins via the N termini. In addition, the sequences reveal that only a few N-terminal residues are critical for the attachment of tailspikes to the adapter protein (Leiman *et al.*, 2007). For phages the attachment of new enzymes with a small adapter protein and the possessing of multiple tail enzymes may confer an evolutionary advantage in environments containing a mixed bacterial population. On the other hand, it could be a disadvantage if the phage is competing with other phages of the same kind (Leiman *et al.*, 2007).

2.2.4 Processive degradation of the polysialic acid capsule

In order to infect bacteria containing a polysialic acid capsule, phages have an endosialidase as a tailspike enzyme. During infection, the capsule-degrading phages create narrow tunnels through the capsule as shown by Bayer and coworkers for *E. coli* K29 using electron microscopy (Bayer *et al.*, 1979). The tunnels are slightly greater in diameter than the phage particle, which indicates that depolymerization of the capsule structure is processive. However, biochemical studies with a purified K1F endosialidase do not support processivity. The distribution of intermediate products indicates that the endoNF binds and cleaves at random sites on the polysialic acid chains (Hallenbeck *et al.*, 1987a).

Using cryoEM reconstructions of the phage particles, Leiman *et al.* have proposed two possible explanations for the conflicting observations of processivity (Leiman *et al.*, 2007). The structure of the endoNF is a trimer that has multiple substrate-binding sites over active sites, and furthermore, each

monomer has two binding sites (Stummeyer *et al.*, 2005). The enzyme needs at least four to eight sugar residues to be functional (Hallenbeck *et al.*, 1987a; Pelkonen *et al.*, 1989; Morley *et al.*, 2009; Schwarzer *et al.*, 2009), which suggests that a cleavable polysialic acid oligomer interacts with the active site and at least one binding site. The lack of processivity reported in biochemical experiments implies that the active site and binding site could not fasten the enzyme properly, and as a result the enzyme was dissociated from the remaining substrate after cleavage (Leiman *et al.*, 2007).

The first explanation proposes that the rates of diffusion of the virion-bound enzyme and the cell-bound polysaccharide are lower *in vivo* than *in vitro* in the experimental assay. This model for the processivity of degradation suggests that even if the enzyme-substrate dissociation occurred *in vivo*, the enzyme and the capsular polysaccharide could remain close enough for efficient rebinding. When a phage begins to degrade a capsule, the substrate concentration increases the nearer the phage comes to the cell surface. This increases the probability of the enzyme rebinding after cleavage. After the beginning of degradation, diffusion of the enzyme will become progressively more restricted. Hence, the processivity will increase as the phage approaches the cell surface (Leiman *et al.*, 2007).

An alternative model proposes co-operativity between the six enzyme molecules of the tailspike that are arranged symmetrically on the phage particle to achieve processivity. As the phage binds to the capsule of a bacterium, a tailspike enzyme associates with a capsular sugar. Before cleavage and dissociation of the first enzyme, a second enzyme binds to a neighboring sugar chain. Complete dissociation from the capsule is unlikely even if an enzyme dissociates from its substrate, because there are six tailspikes on the phage. The six tailspikes may function coordinately, even though they are not in direct contact. They may be able to communicate through common attachment to the phage tail. During an enzymatic cycle, a tailspike could undergo a conformational change, which is transmitted through the tail to the neighboring tailspikes, which increases the affinity of the neighbor for its substrate. Co-operation between tailspikes could help them to function sequentially and the phage would then degrade polysialic acid to reach the cell surface (Leiman *et al.*, 2007).

The phage will stop degradation of the capsule when the polysialic acid chains become too short for digestion. The phage must still remain bound to the sugar to maintain infection because the polysialic acid-binding activity is necessary for the recognition of the receptor. In the infection of mutant bacteria with sparse capsules, the bacteriophage had no need to degrade the capsule, and the cleaving activity had an inhibitory effect on the infection (Pelkonen *et al.*, 1992).

2.2.5 Substrate size dependency of the endosialidase

Recently the substrate specificity of the endoNF to cleave the α2,8 linked Neu5Ac chain has been studied using ^{1}H NMR, and corroborated with anion-

exchange chromatography. The results showed that an oligomer of four sialic acid units (DP4) was cleaved by the endosialidase to monomer and DP3 (Figure 5), but DP2 and DP3 were not cleaved (Schwarzer *et al.*, 2009). Thus, DP4 is the minimum length of sialic acid residues needed for cleavage with endosialidase F.

Substrate cleaving with the endoNF has also been studied with synthetic oligomeric trifluoromethylumbelliferyl (TFMU) sialosides, and it was observed that at least three units are needed on the non-reducing side (distal fragment) for cleavage (Morley *et al.*, 2009). This is in agreement with findings that have been reported for oligosialosides of the PK1A enzyme (Finne *et al.*, 1985; Hallenbeck *et al.*, 1987a; Kataoka *et al.*, 2006) and with results from an ^{1}H NMR study on the endoNF (Schwarzer *et al.*, 2009). The minimum substrate binding sizes required for cleavage may also, however, show differences between endosialidases.

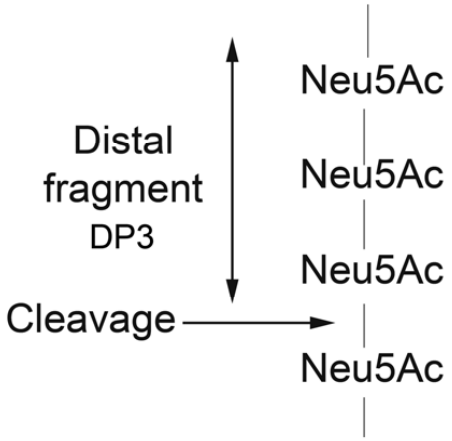

Figure 5. The substrate cleaving specificity of the endosialidase F. The linear α2,8 Neu5Ac chain is cleaved by the endosialidase to a distal DP3 unit and a monomer.

2.2.6 The enzymic mechanism of endosialidases

The active site of endoNF lacks many residues that are conserved in exosialidases, which suggests that endosialidases may have a catalytic mechanism different from other sialidases. Recent studies using ^{1}H-NMR and a synthetic TFMU sialotrioside have demonstrated that endoNF is an inverting sialidase (Morley *et al.*, 2009). The endosialidase thus differs from all other wild type sialidases which are retaining glycosidases. Inverting enzymes use two enzymic residues, typically carboxylate residues that act as an acid and a base. Hydrolysis occurs by an acid/base-catalyzed double-displacement mechanism. In the endosialidase, these residues are proposed to correspond to the sialic

acid carboxylate acting as the general base and most likely E581 as the general acid.

2.3 Detection methods for polysialic acid

2.3.1 The use of an active endosialidase

A prokaryotic-derived endosialidase recognizes and degrades polysialic acid, but in eukaryotes a corresponding enzyme has not been found. A bacteriophage-derived endosialidase has been used for removing polysialic acid from neonatal neuronal membranes (Vimr *et al.*, 1984) and it has been shown to rapidly and specifically degrade polysialic acid under physiological conditions (Finne *et al.*, 1985; Rutishauser *et al.*, 1985). An endosialidase can be used as a tool to study polysialic acid. It provides the ability to indirectly detect or degrade polysialic acid under many experimental and physiological conditions. An endosialidase is often used together with other methods in the detection of polysialic acid, where specific binding is confirmed by cleaving polysialic acid with an active endosialidase.

2.3.2 Examples of antibody based detection of polysialic acid

The need to create an effective vaccine against *N. meningococci* B has pushed forward the development of polysialic acid antibodies and antibody mimics. The poor immunogenecity of purified polysialic acid has made it difficult to produce antibodies against polysialic acid, but ultimately both polyclonal and monoclonal antibodies have been developed against it. Some examples of frequently used or otherwise interesting antibodies are listed in Table 2.

The H.46 polyclonal antibody (Sarff *et al.*, 1975; Allen *et al.*, 1982) was produced in a single horse against *Neisseria meningitidis* B, and it is therefore not easily available. One of the most frequently used antibodies is monoclonal antibody 735. It was raised in autoimmune New Zealand black mice against group B meningococcal antigen (Frosch *et al.*, 1985). Both of these antibodies specifically recognize long chains of α2,8-linked sialic acid, 735 needs at least eight and H.46 about ten residues for binding. Both of them have been used for the detection of polysialic acid in eukaryotes (Finne *et al.*, 1987). The 2-2B mouse monoclonal antibody was also produced against the capsule of group B *N. meningitidis* and was used in the staining of polysialic acid in cell lines of neural origin (Mandrell *et al.*, 1982; Rougon *et al.*, 1986).

Mouse monoclonal antibody OL.28 was obtained when BALB/c mice were immunized with primary cultures of newborn rat oligodendrocytes. This antibody also stained the cell surface of rat basophilic leukemia cells and MCF7 human breast cancer cells, and the recognition of polysialylated proteins was confirmed by monoclonal antibody 735 (Martersteck *et al.*, 1996). Both monoclonal

antibodies, 12E3 (Seki *et al.*, 1991; Seki *et al.*, 1993) and 5A5 (Dodd *et al.*, 1988; Acheson *et al.*, 1991), were obtained using a rat brain derived-antigen and both have been used in immunohistochemical studies.

IgM^{NOV} is a macroglobulin which was purified from an 81-year-old macroglobulinemia patient (NOV) (Kabat *et al.*, 1986; Kabat *et al.*, 1988). The staining pattern in the tissue sections with IgM^{NOV} and 735 were different, which results from the crossreactivity of IgM^{NOV} with polynucleotides and DNA (Husmann *et al.*, 1990). Crossreactions were suggested to be due to a similar spatial distribution of charges in the carboxyl groups of α2,8-polysialic acid and the phosphates of denatured DNA or polynucleotides (Kabat *et al.*, 1988).

Monoclonal antibody 1E6 specifically recognizes the α2,8-disialic acid glycotope. A synthetic polymer conjugated to two Neu5Ac units was used as an immunogen to immunize the New Zealand Black mice (Sato *et al.*, 2000). IgM S2-566 is another disialic acid-recognizing monoclonal antibody which recognizes the Neu5Ac-α2,8-Neu5Ac-α2,3-Gal- glycotope, where the Gal residue is required. The S2-566 antibody was used in Western blot analysis of brain glycoproteins (Sato *et al.*, 2000).

Table 2. Anti-oligo/polysialic acid antibodies and their recognition specificity dependence on the degree of polymerization (DP).

Antibody	Origin/type	Immunizing antigen	Recognition specificity (DP)
H.46	horse polyclonal	Inactivated Men B bacteria	≥8
IgM^{NOV}	human monoclonal	From macroglobulinemia patient	–
735	mouse monoclonal	Men B bacteria	≥11
12E3	mouse monoclonal	Embryonic rat forebrain	≥5
5A5	mouse monoclonal	Embryonic rat forebrain membranes	≥3
2-2B	mouse monoclonal	Men B bacteria	≥4
OL.28	mouse monoclonal	Newborn rat oligodendrocyte	≥4
S2-566	mouse monoclonal	Human ganglioside GD3	2
1E6	mouse monoclonal	$(Neu5Ac)_2$ bearing artificial polymer	2

2.3.3 A phage-based method for the detection of polysialic acid

A phage-based detection method has been described which utilizes a spontaneous host-range mutant of the PK1A phage that has totally lost its catalytic activity, but still retained polysialic acid-binding activity (Pelkonen 1990; Pelkonen *et al.*, 1992). In this method, the phage mutant is used for recognizing polysialic acid, and the bound phage is detected with an anti-phage antibody followed by a peroxidase-conjugated secondary antibody (Aalto *et al.*, 2001). The mutant phage recognizes both bacterial and eukaryotic polysialic acid, but the method includes multiple steps and time-consuming propagation of phages. However, this study gave an indication that reagents derived from bacteriophages could be used for the specific detection of polysialic acid.

2.3.4 Chemical and chromatographic methods for polysialic acid analysis

Polysialic acid has been analyzed with methods such as thin-layer chromatography (Finne *et al.*, 1985), thin-layer chromatography combined with mild acid hydrolysis (Kitazume *et al.*, 1992), ion-exchange chromatography of NaB^3H_4-labeled sialic acid oligomers (Finne *et al.*, 1985), gel electroforesis of capsular polysialic acid and its fragments (Pelkonen *et al.*, 1988), immunoaffinity chromatography of polymers with DP≥10 (Halberstadt *et al.*, 1993) and high performance capillary electroforesis (HPCE) (Cheng *et al.*, 1998).

In NMR, the chemical shifts of the carbons and protons of the sialic acid residues of the oligo/polysialic acid can be used for the detection of the ^{13}C NMR and ^{1}H NMR spectra, respectively (Michon *et al.*, 1987). In a methylation analysis, neuraminic acid is methylated and quantitated using gas chromatography combined with mass spectrometry (Rauvala *et al.*, 1977). With this method, glycoproteins from different tissues were shown to contain disialyl units (Finne *et al.*, 1977). Many other chemical and chromatographic methods are now available for oligo/polysialic acid detection.

High-performance liquid chromatography (HPLC) (Hallenbeck *et al.*, 1987b) has been used for the detection of polysialic acid, and more recently, it has been developed to become an efficient method of polysialic acid analysis. This method includes applications such as the analysis of sialic acid dimers with high-performance anion-exchange cromatography with pulsed amperometric detection (HPAEC-PAD) (Inoue *et al.*, 2001a). HPAEC-PAD has also been used in tandem with HPLC equipped with a fluorescence detector (Inoue *et al.*, 2000).

Derivatization with 1,2-diamino-4,5-methylenedioxybenzene /high-performance liquid chromatography with fluorescence detection (DMB/HPLC-FD) could be used as a diagnostic assay for detecting oligo/polysialic acid from tissue homogenates and cells, and also for determination of its degree of polymerization. In this method, polysialic acid-containing samples are reacted with fluorogenic DMB followed by separation by HPLC and a fluorometric

detection (Inoue *et al.*, 2001b). The advantage of this method is that both the degree of polymerization and the relative amount of polysialic acid can be estimated simultaneously (Inoue *et al.*, 2001b). The DMB/HPLC-FD method can also be applied to the detection of oligo/polysialic acid samples in solution and, in addition, on membrane blots (Sato *et al.*, 1999).

Fluorometric C_7/C_9 detection is a sensitive chemical method for the determination of the degree of polymerization of oligo/polysialic acid at the microgram level (Sato *et al.*, 1998; Sato *et al.*, 1999). The method is based on the selective oxidation of the terminal sialic acid unit to its C_7 derivate. The analysis includes oxidation/reduction, acid hydrolysis, DMB derivatization to C^7(Neu5Ac)-DMB and C^9(Neu5Ac)-DMB and quantitation with HPLC. This technique can also be applied to an oligo/polysialic acid analysis on intact membranes. However, this method is not applicable for modified sialic acid structures (Inoue *et al.*, 2003).

HPLC profiling has been used for analysis of the degree of polymerization of polysialic acid chains on NCAM. A preceding endo-β-galactosidase treatment releases polysialic acid from NCAM before subsequent profiling with HPLC. Using this approach, acid hydrolysis, which may lead to some fragmentation of the polysialic acid chains, can be avoided (Nakata *et al.*, 2005).

Polysialic acid is difficult to analyze with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) because it includes multiple acidic groups. These groups should be masked before the analysis by lactonization (see structure in Figure 3 on page 15). This method is also applicable for fluorescently labeled oligo/polysialic acid derivates, such as DMB derivates. Compared to DMB/HPLC-FD, MALDI-TOF-MS is more sensitive, but requires purified samples (Galuska *et al.*, 2007).

The polysialic acid detection methods discussed here are summarized in Table 3. Only a few methods are applicable to the detection of small amounts of oligo/polysialic acid (less than 1 µg): fluorometric anion-exchange HPLC analysis, fluorometric C_7/C_9 detection, MALDI-TOF-MS, and western blotting using specific antibodies and enzymes.

Table 3. Chemical and chromatographic methods for polysialic acid analysis.

Method	Reference
Thin-layer chromatography	Finne *et al.*, 1985
Thin-layer chromatography combined with mild acid hydrolysis	Kitazume *et al.*, 1992
Ion-exchange chromatography of ^{3}H-labeled sialic acid oligomers	Finne *et al.*, 1985
Gel electrophoresis of ^{3}H-labelled sialic acid oligo and polymers	Pelkonen *et al.*, 1988
Immunoaffinity chromatography	Halberstadt *et al.*, 1993
High-performance capillary electrophoresis	Cheng *et al.*, 1998
Nuclear magnetic resonance	Michon *et al.*, 1987
Methylation analysis	Rauvala *et al.*, 1977
High-performance liquid chromatography	Hallenbeck *et al.*, 1987b
High-performance anion-exchange cromatography with a pulsed amperometric detector	Inoue *et al.*, 2001a
• in tandem with high-performance liquid chromatography with fluorescence detection	Inoue *et al.*, 2000
Derivatization with 1,2-diamino-4,5-methylenedioxybenzene / high-performance liquid chromatography with fluorescence detection	Sato *et al.*, 1999
Fluorometric C_7/C_9 detection	Sato *et al.*, 1998
High-performance liquid chromatography profiling after endo-β-galactosidase treatment	Nakata *et al.*, 2005
Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry	Galuska *et al.*, 2007

3 AIMS OF THE PRESENT STUDY

Polysialic acid is involved in many biological events, including development, malignancy and infectious processes. Therefore, there is a need for efficient analytical methods for its detection. Polysialic acid is poorly immunogenic and the preparation of high affinity antibodies has been challenging. It has been reported previously that bacteriophages with a defect in their catalytic activity of endosialidase could be used as potential reagents for the detection of polysialic acid. The aim of this thesis was therefore to investigate this possibility in more detail.

The specific aims were:

- to study wheather an inactive endosialidase could be utilized for the construction of an efficient detection method for polysialic acid

- to identify the differences in sequences between a native and mutant endosialidase and by that means seek to elucidate the structural basis of the inactivity of the enzyme

- to test the applicability of the developed method for the detection of polysialic acid in an experimental bacterial infection model and in neuroblastoma

4 MATERIALS AND METHODS

The detailed descriptions of the materials and methods used in the present study are given in the original publications I-IV.

4.1 Bacterial strains, phages and plasmids

The bacterial strains, bacteriophages and plasmids are listed in Tables 4, 5 and 6, respectively.

Table 4. Bacterial strains.

Bacterial strain	Characteristics	Source/reference
Escherichia coli		
IH3088	The host strain of native bacteriophage PK1A and PK1E (I, II)	Korhonen *et al.*, 1985
IHE3083	K2 capsule (I,II)	Korhonen *et al.*, 1985
EH954	Derived from rough *E. coli* IH3088 (II)	Pelkonen *et al.*, 1987
EH1008	Derived from rough *E. coli* IH3088 (II)	Pelkonen *et al.*, 1987
M15 (PREP4)	Expression strain (I,II)	Qiagen
A192	From a patient with septicemia (III)	Achtman *et al.*, 1983
A192PP	Complement-resistant strain, enhanced virulence(III)	Mushtaq *et al.*, 2004
A192PPK$^-$	Stable, capsule-free mutant (III)	III
EV36	K127/K1 hybrid, produces a non-acetylated K1 capsule (III)	Vimr *et al.*, 1985
BL21(DE3)	Expression strain (III)	Novagen
Neisseria meningitidis		
NCTC10025	Clinical isolate, serotype A (I)	E. Eerola, U. Turku
NCTC10026	Clinical isolate, serotype B (I)	E. Eerola, U. Turku
NCTC8554	Clinical isolate, serotype C (I)	E. Eerola, U. Turku
Moraxella nonliquefaciens		
KK987/84	Clinical isolate, polysialic acid-positive strain (I)	H. Järvinen, THL, Turku
EF10057	Clinical isolate, polysialic acid-negative strain (I)	E. Eerola, U. Turku
Mannheimia (Pasteurella) haemolytica		
KU 201/83	Capsule type A2 (I)	Aalto *et al.*, 2001
KU 301/83	Capsule type A2 (I)	Aalto *et al.*, 2001
KU 363/84	polysialic acid-negative strain (I)	Aalto *et al.*, 2001

Table 5. Bacteriophages and their mutants.

Bacteriophage	Description	Source/reference
PK1A	Native (II)	Gross *et al.*, 1977
PK1A2	Mutant of PK1A (I, II)	Pelkonen *et al.*, 1992
PK1A5	Mutant of PK1A (II)	Pelkonen *et al.*, 1992
PK1A8	Mutant of PK1A (II)	Pelkonen *et al.*, 1992
PK1E	Native (II, III)	T. Cheasty, Health Protection Agency, UK
PK1E3	Mutant of PK1E (II)	Pelkonen *et al.*, 1992

Table 6. Plasmids.

Plasmid	Characteristics	Source/reference
pEGFP	EGFP obtained for cloning (I)	Clontech
pQE-31	Cloning vector (I)	Qiagen
pOP1	*Yersinia enterocolitica* adhesin A, yadA stalk for cloning (I)	Nummelin *et al.*, 2002

4.2 Antibodies and NCAM

The sources of the antibodies used in the immunohistochemical analyses as well as NCAMs are listed in Table 7.

Table 7. Primary antibodies and special reagents.

Antibody	Antigen	Source
Mouse monoclonal antibody 735	polysialic acid (I)	R. Gerardy-Schahn, Frosch *et al.*, 1985
Rabbit polyclonal antibody	*E. coli* O18 surface LPS (III)	T. Cheasty, Health Protection Agency, UK
Mouse monoclonal IgG2b antibody Ab8064	O-acetylated form of K1 (III)	Abcam
Polysialylated embryonic NCAM	(I)	R. Gerardy-Schahn, Hannover
Adult NCAM	(I)	R. Gerardy-Schahn, Hannover

4.3 General laboratory methods

The general laboratory methods used are summarized in Table 8.

Table 8. General laboratory methods.

Method	Described in
Isolation of phage DNA	I, II
Recombinant DNA techniques	I,II
PCR	I,II
DNA sequencing and analysis	I,II
Electroporation	I,II
Bacteriophage propagation	II
Surface plasmon resonance assay	I
Gel filtration	I
Protein production and purification	I, II, III, IV
Protein homology modeling	II
Enzyme activity assay	I, II
Immunoblot analyses	I
Electophoresis	I, II
Cell culture	I, II,IV
Preparation of tissue sections	I, II, III
Immunohistochemical staining	III
Light microscopy	III
Fluorescence counting	I
Fluorescence microscopy	I, II, III, IV
Confocal microscopy	I, II, III, IV
Statistical analysis	III

4.4 Construction and purification of fusion proteins (I)

Endosialidases of catalytically active or inactive PK1A bacteriopages were used for the construction of endosialidase–green fuorescent protein (GFP) fusion proteins. To increase the amount of soluble protein, a part of the *Yersinia enterocolitica* adhesin (yadA) stalk was used as a spacer between the endosialidase and GFP. Truncated or mutated derivatives were made by PCR and expressed as fusion proteins. All constructs were inspected by sequencing and endosialidase activity assays. Constructs were electroporated to the expression strain and expressed as N-terminal histidine-tagged fusion proteins. Proteins were purified using immobilized Ni-affinity-chromatograghy. Sodium dodecyl sulfate-polyacrylamide gel electroforesis (SDS-PAGE) was used for analyzing the purity of the protein products.

4.5 Detection of polysialic acid with a fusion protein (I, II, III, IV)

The fusion protein with an inactivated endosialidase was used for the detection of polysialic acid in cells, bacteria, paraffin-embedded tissue sections, frozen sections, immunoblots and fluorescence counting. After blocking of the non-

specific binding, the fusion protein was added to the specimens and incubated for one hour in the dark. After washing, the specimens used for the microscopy were stained with the appropriate stains and mounted on slides using a medium containing an anti-fading reagent. The control cell lines known to express polysialic acid on their cell surface were baby hamster kidney cells (BHK-21 [C-13] ATCC: CCL-10) and human neuroblastoma cells (SH-SY5Y ECACC: 94030304). The tissue samples were obtained with the approval of the Joint Ethical Committee of the University Hospital of Turku and the University of Turku.

4.6 An animal model for **E. coli** *K1 infection (III)*

The *E. coli* infection model was adapted from the rat model used by Glode *et al*. (Glode *et al.*, 1977) and Pluschke *et al*. (Pluschke *et al.*, 1983). The newborn rats were fed with *E. coli* A192PP, and the progression of the bacterial infection was followed. After sacrifice, organs were collected and divided into two portions, one for the calculation of *E. coli* K1, and the other for preparation of paraffin-embebbed sections. These experiments were carried out in P. W. Taylor's laboratory in London, UK.

5 RESULTS AND DISCUSSION

5.1 The sequence of the PK1A endosialidase (II)

The endosialidase gene sequenced in this study encodes a tailspike of the PK1A phage. Its length is 2436 bp in the native form and it encodes a polypeptide of 811 amino acid residues. The estimated molecular weight according to the amino acid sequence is 89 kDa (II, Figure 1), but after proteolytic processing the molecular weight of the mature protein monomer is 76 kDa (II, Figure 2). When compared to other endosialidases the PK1A enzyme is similar in size to that of PK1E, and has exactly the same length as the PK1-5 endosialidase (Scholl *et al.*, 2001). The PK1F polypeptide is 253 amino acid residues longer than the PK1A endosialidase (Mühlenhoff *et al.*, 2003). The amino acid sequence of the translated native endosialidase PK1A is very similar to those of PK1E (Gerardy-Schahn *et al.*, 1995; Long *et al.*, 1995) and K1-5 (Scholl *et al.*, 2001), having a 90% and 89% identity, respectively.

The endosialidase protein contains two sialidase (neuraminidase) motifs, Ser-Xaa-Asp-Xaa-Gly-Xaa-Thr-Trp (II, Figure 1), which are typical of glycosyl hydrolases. The Asp-motifs are supposed to maintain the correct structural folding and affect the solubility properties of the enzyme (Crennell *et al.*, 1994; Gaskell *et al.*, 1995; Chien *et al.*, 1996; Leggate *et al.*, 2002). When performing search in the database of protein families, an eight-element fingerprint provides a signature for the glycosylhydrolase family 58. Moreover, the endosialidases of K1 phages K1E, K1-5, K1F and prophage CUS-3 belong to the same family. The signatures of the PK1A endosialidase (Figure 6) are in amino acid sequence positions 104–129, 169–198, 288–312, 358–385, 436–463, 492–517, 598–625, and 630–658 (http://www.ebi.ac.uk/interpro/ and http://www.cazy.org/fam/GH58.html) (Brandi L. Cantarel *et al.*, 2009). Positions 38–45 of the PK1A endosialidase amino acid sequence contain (Ala or Gly)-Xaa-Xaa-Xaa-Xaa-Gly-Lys-(Ser or Thr) (Figure 6), which is a well-conserved motif of ATP/GTP-binding proteins called “A” consensus sequence or P-loop (Walker *et al.*, 1982; Saraste *et al.*, 1990). The putative PK1A endosialidase promoter sequence (Figure 6) is exactly the same as predicted for the K1-5 consensus promoter (Scholl *et al.*, 2004), which has sequence similarity with the consensus promoter of SP6 (Scholl *et al.*, 2004). Similarly to the sequence of PK1E endosialidase (Leggate *et al.*, 2002), that of the PK1A endosialidase has two putative Shine-Dalgarno (S-D) ribosomal binding sites upstream from the start codon (Figure 6).

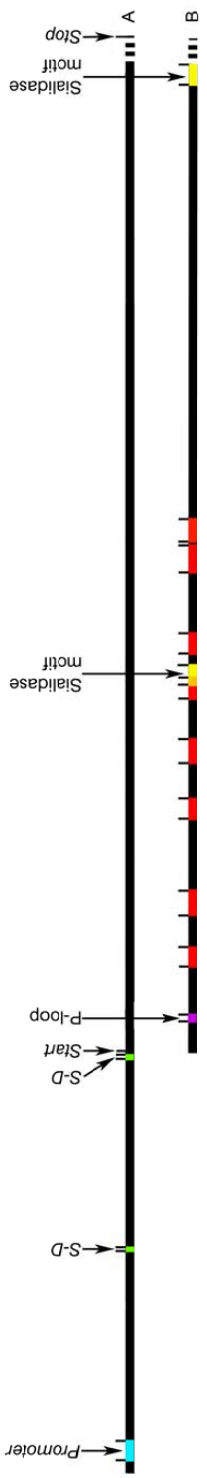

Figure 6. A schematic picture of the PK1A endosialidase (A) DNA and (B) protein sequence. Characteristics of the PK1A endosialidase sequences are indicated as follows: in the DNA sequence, the putative promoter region in blue and Shine-Dalgarno (S-D) sequences in green; in the protein sequence, P-loop in purple, the sialidase motifs in yellow and the signature fingerprints of the sequence in red. An area where a sialidase motif and a signature fingerprint is illustrated in orange.

5.2 Construction of a fusion protein for polysialic acid detection (I, II, IV)

Spontaneous mutants of *E. coli* K1 bacteriophages which have lost the polysialic acid cleaving activity of their endosialidase but retained the binding activity (Pelkonen *et al.*, 1992) have been used for the detection of polysialic acid from both bacteria and eukaryotic cells (Aalto *et al.*, 2001). Thus, in order to investigate whether inactive endosialidase could be used as the basis of a polysialic acid detection reagent, the endosialidase gene of the mutant PK1A2 phage was used for the construction of an endosialidase–GFP fusion protein. Direct fusion of the endosialidase and GFP genes and expression in *E. coli* produced an essentially insoluble protein. To increase the proteins in the soluble form, a spacer was added between the endosialidase and the GFP. A repeat part of the *Yersinia enterocolitica* adhesin (yadA) stalk (Hoiczyk *et al.*, 2000) was chosen as the spacer (Figure 7). A series of truncated forms of the yadA stalk were tested, but the longest form of the stalk was needed to yield large amounts of a stable protein in a soluble form (I, Figure 1, panels A-C).

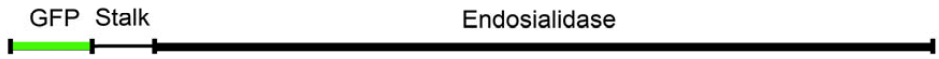

Figure 7. A schematic illustration of the fusion protein construct.

As a control, a fusion protein containing the catalytically active form of the endosialidase was made. When the BHK-21 or SH-SY5Y cells, known to express surface polysialic acid, were stained with fusion proteins, only the fusion protein with the inactive form of the enzyme stained the cells (I, Figure 3; II, Figure 5; IV, Figure 3A). The endosialidase of the catalytically active fusion protein degraded the polysialic acid from the cell surface as confirmed by post-staining either with the fusion protein with an inactive endosialidase or monoclonal antibody 735 to polysialic acid (I, Figure 3, panels B and C). Thus, catalytic inactivation was a prerequisite for the detection of polysialic acid. As further controls, the staining could be inhibited by the addition of free polysialic acid (I, Figure 3D; IV, Figure 3C), or pretreatment with an active endosialidase (II, Figure 6B). To further demonstrate the specificity of the fusion protein, the staining patterns were compared with those obtained with monoclonal antibody 735, and found to be essentially the same (I, Figure 3, panels A and E) and (Figure 8).

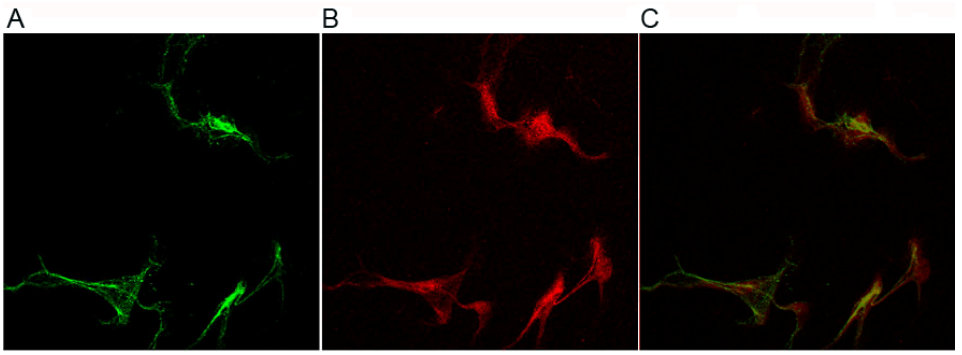

Figure 8. Expression of polysialic acid on the BHK 21 cells detected with the (A) fusion protein and (B) monoclonal antibody 735. (C) Overlay image of (A) and (B) indentified colocalized staining patterns of the fusion protein and monoclonal antibody 735.

The binding affinity between the fusion protein and polysialic acid was determined using a biosensor technique based on surface plasmon resonance. The binding of polysialic acid (colominic acid) to the fusion protein was dose-dependent over a polysialic acid concentration range of 1.6 nM–1 µM (I, Figure 2A), and the binding constant was determined by equilibrium binding of the Scatchard plot analysis (I, Figure 2B). A curved shape of the Scatchard plot is typical for bivalent interactions (Pellequer *et al.*, 1993; MacKenzie *et al.*, 1996). The dissociation constant K_D values calculated separately for the two phases of the curve were 5.0×10^{-9} M and 33×10^{-9} M and the K_D (average) 19×10^{-9} M. These values are higher than the corresponding values of 10^{-6} to 10^{-8} determined for several anti-carbohydrate antibodies (Pellequer *et al.*, 1993) and comparable with the values of the best-known polysialic acid antibody mAb 735 (K_D 2.5×10^{-9} M and 11×10^{-9} M, average 7×10^{-9} M) (Häyrinen *et al.*, 2002). The enzyme–substrate interactions are comparable to lectin–carbohydrate interactions, where the K_D values usually range from millimolar to micromolar. Lectins are widely used as molecular markers.

The oligomeric state of the fusion protein was studied by gel filtration. The fusion protein was eluted as a single peak (I, Figure 1E) only slightly earlier than the calculated molecular mass (360 kDa) of the fusion protein containing the endosialidase in trimeric form. This is consistent with an earlier observation for the native endosialidase (Mühlenhoff *et al.*, 2003).

5.3 Structural basis of endosialidase inactivation (II)

5.3.1 The catalytic and binding activities of endosialidases (II)

Mutants of the PK1A bacteriophages of spontaneous origin were selected by their ability to infect *E. coli* with a sparse K1 capsule (Pelkonen *et al.*, 1992). The sequencing of the endosialidase genes of the three PK1A mutants revealed that they were equal in size to the wild type gene. Each of the three mutants had only one to three amino acid substitutions at different locations (II, Table 3). The substitution R614G in PK1A5 and PK1A8 was a conserved normal allele present in an endosialidase of other wild-type phages, PK1E (Gerardy-Schahn *et al.*, 1995; Long *et al.*, 1995), PK1-5 (Scholl *et al.*, 2001) and PK1F (Mühlenhoff *et al.*, 2003), displaying full enzymic activity.

The endosialidases of mutant phages PK1A2 and PK1A5 were revealed to be catalytically inactive, whereas there was residual activity in the PK1A8 and PK1E3 mutants (II, Figure 3). The results are consistent with the published qualitative analysis of the enzyme activities of these phages (Pelkonen *et al.*, 1992). The endosialidases of mutant PK1A2 and native PK1A phages differ only by two amino acid residues. The role of these two point mutations was studied by back-mutating the gene individually at both locations, and expressing the mutants with single amino acid substitution as GFP-fusion proteins. Both back-mutated constructs were able to degrade polysialic acid, but to a lesser extent than the construct with the native endosialidase (II, Figure 4). Thus, both of the two amino acid substitutions were needed for complete inactivation of the enzyme. Some earlier results reported for engineered alanine-substitutions in endosialidases are conflicting (Leggate *et al.*, 2002; Stummeyer *et al.*, 2005; Morley *et al.*, 2009). One replacement alone (E581A) of three critical residues in the putative active site of the PK1F endosialidase decreased its activity by about 95%, but double-mutated enzymes were inactive yet capable of binding polysialic acid (Stummeyer *et al.*, 2005). Using a synthetic TFMU oligosialoside as a substrate for endosialidase PK1F, mutation E581A was reported to result in total inactivation of the enzyme (Morley *et al.*, 2009). The conflicting result could be explained by a preferential action of the endosialidase towards oligosialosides, as described earlier (Hallenbeck *et al.*, 1987a), or an inaccuracy in the assay method used in the earlier study (Morley *et al.*, 2009). In the endosialidase of PK1E, a replacement of D138A was reported to reduce the catalytic activity by 80% (Leggate *et al.*, 2002). The mutated amino acids in the sequence of the endosialidase are listed in Table 9.

Table 9. Mutations and amino acid positions in the sequences of PK1A, PK1F and PK1E endosialidases. The sequences were aligned with the ClustalW2 program (Thompson *et al.*, 1994) (http://www.ebi.ac.uk/Tools/) using GeneBank accession numbers EF507428, AM084414 and AM084415 for the endosialidases of PK1A, PK1F and PK1E, respectively.

Position/Mutation in the PK1A sequence	**Position in the PK1F sequence**	**Position in the PK1E sequence**
D138	D348	D138
E371	E581	E371
R386	R596	R386
R437	R647	R437
H417Y	H627	H417
N489D	N699	N489

The influence of individual amino acid substitutions of the molecules on the polysialic acid binding activity was determined, and their binding to polysialic acid-containing cells was studied as GFP-fusion proteins. Only those derivatives with a catalytically inactive endosialidase bound to the cells, whereas no binding was observed with the partially active endosialidases (II, Figure 5). Loss of binding was due to degradation of the polysialic acid on the surface of the cells.

In order to investigate the effects of terminal deletions on the binding and catalysis, N- and C-terminal deletions were introduced to the wild type and the mutant PK1A endosialidases (II, Table 4; Figure 9). The catalytic activity of the wild-type PK1A endosialidase was sensitive to deletions; only the first N-terminal deletion derivate (∆1–47) was catalytically active, and all C-terminal deletion derivatives were inactive. Deletion derivative (∆1–107) of the wild type PK1A enzyme was catalytically inactive, but had binding activity to polysialic acid (II, Figure 6). In the case of the PK1A mutant endosialidase, all derivatives were inactive, but the first two N-terminal deletion derivatives (∆1–47 and ∆1–107) were able to bind polysialic acid at a low level. As a difference to the wild type truncated derivative (∆1–107), the same inactive PK1A2 mutant endosialidase deletion derivative had increased expression levels. All of the more truncated endosialidase derivatives were insoluble. Based on a comparison with the PK1F structure (Stummeyer *et al.*, 2005), it seems that the deletions producing insoluble enzymes either removed essential parts of the central (catalytic) domain, or affected the C-terminal portion, which is essential for the trimerisation of the molecule (Mühlenhoff *et al.*, 2003).

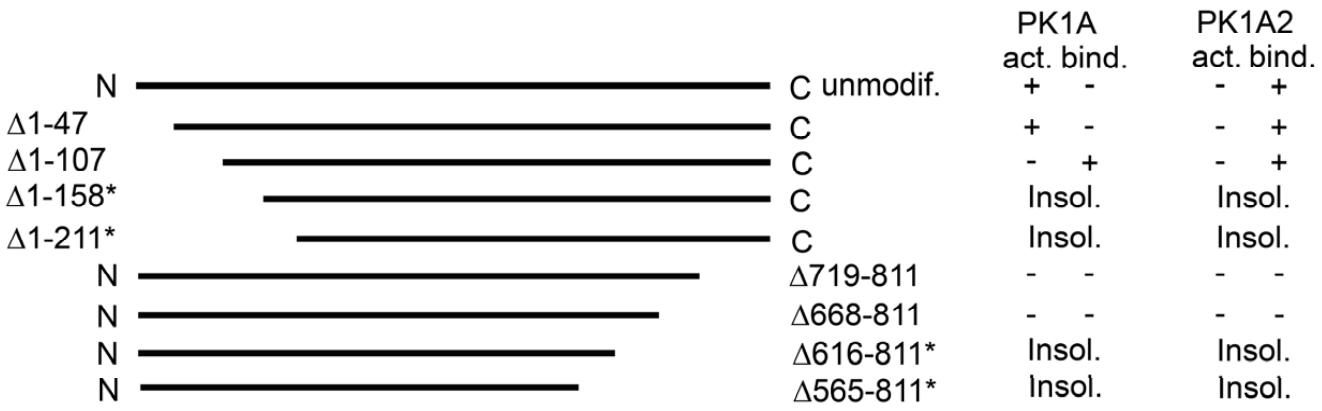

Figure 9. A schematic presentation of the terminal deletions introduced in the protein sequences of the wild-type PK1A and mutant PK1A2 endosialidase. Catalytic (act.) and binding (bind.) activities of the derivates were assayed as GFP-fusion proteins, and described with + (binds) and – (no binding). The Insoluble deletion derivates are marked by Insol.

5.3.2 Structural modeling of the PK1A endosialidase (II)

The first crystal structure of an endosialidase was reported in 2005 for the endosialidase of the PK1F phage and its complex with oligomeric sialic acid (Stummeyer *et al.*, 2005). The PK1F sequence contains an amino-terminal extension which the PK1A, PK1E, and K1-5 endosialidases do not have, and this domain is thought to mediate the binding of the PK1F endosialidase to the phage head (Petter *et al.*, 1993). The central regions are highly conserved and form the active parts of the enzymes (Long *et al.*, 1995). They include two Asp-box motifs, which have a sequence similarity with exosialidases. All of the four enzymes are post-translationally processed in their C-termini (Petter *et al.*, 1993; Gerardy-Schahn *et al.*, 1995; Long *et al.*, 1995; Scholl *et al.*, 2001; Mühlenhoff *et al.*, 2003).

The sequence alignment of the PK1A and PK1F endosialidases indicates homology of the proteins. In PK1F, 663 out of 666 residues were aligned with the PK1A residues, and 555 of these pairs were identical. Residues 1–37 of PK1A did not align with any residue of PK1F. The crystal structure of the PK1F endosialidase lacks amino-terminal residues. However, the aligned sections of the PK1A and PK1F sequences have 83% identity. Thus, the PK1F endosialidase structure was used to build a homology-based structural model for residues 38–706 of the PK1A endosialidase by using the MALIGN algoritm (Johnson *et al.*, 1993) in the program Bodil (Lehtonen *et al.*, 2004)

The high identity of the homologs makes it possible to study the point mutations observed in the sequence of the PK1A endosialidase in the structural model. The distribution of conserved residues within the alignment supports this. The *β*-propeller domain contains the active site of the enzyme, and is well conserved; the sequences are 93% identical. Furthermore, the sequence of the *β*-barrel domain is 100% identical. Thus, the differences are located mostly in the tailspike domain and in the amino-terminal fragment, which forms a cap structure for the *β*-propeller domain.

Four spontaneous point mutations of the PK1A (W118C, H332N, H417Y and N489D) and one mutant of the PK1E3 (A370E) endosialidase with reduced enzymic activities are located at or near the putative active site, where the degradation of polysialic acid has been proposed to occur (II, Figure 7). None of these amino acid substitutions corresponds to any of the three residues (E371, R386, and R437 in PK1A) suggested to be in the active site of PK1F. Furthermore, the four residues are all conserved between the PK1A and PK1F endosialidases. The amino acid positions of three residues and mutations H417Y and N489Y in the sequence of PK1F endosialidase are listed above in Table 9.

Mutations H417Y and N489Y in the PK1A2 endosialidase (II, Table 3) are fundamentals for the functioning of the fusion protein. Histidine 417 is buried and not in a direct position to bind the substrate (Figure 10). The replacement of histidine with tyrosine could have an indirect effect, when the surrounding residues adapt to the change of size, shape and hydrogen bonding tendencies. N489 is located on the edge of the putative active site (Figure 10). N489 forms a part of a ridge that is between the putative active site and the polysialic acid binding site formed by another chain of the trimer (Stummeyer *et al.*, 2005). Thus, it is possible that when the polysialic acid chain is bound into the active site, the chain interacts with asparagine at position 489. However, when the asparagine has mutated into aspartic acid, the charge of the side chain has changed so that the carbohydrate chain cannot form all the required interactions.

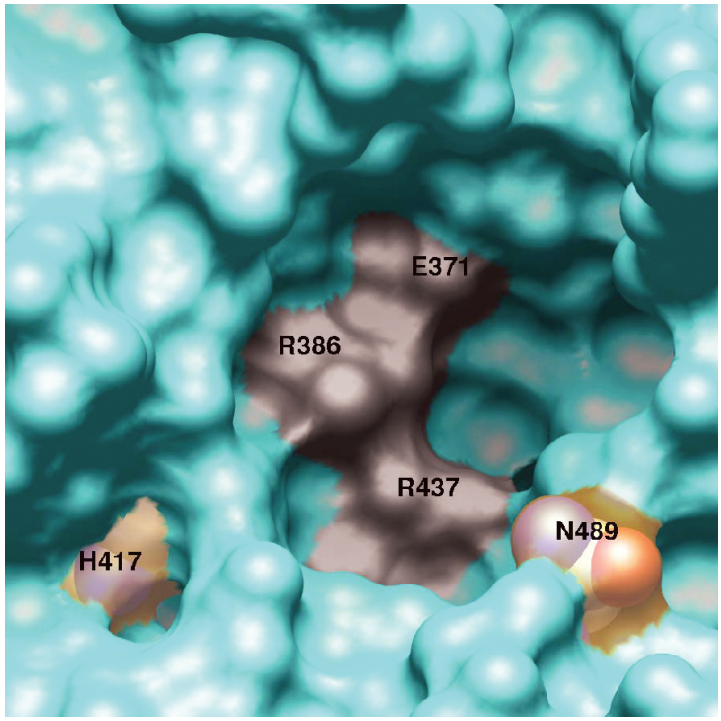

Figure 10. The location of the mutations (H417 and N489) important for the inactivation of the PK1A endosialidase. The three residues (E371, R386 and R437 in PK1A) reported as putative active site residues of PK1F are indicated in grey. The image is made using the MALIGN algorithm (Johnson *et al.*, 1993) in the program Bodil (Lehtonen *et al.*, 2004). Courtesy of Jukka Lehtonen.

5.4 Detection of polysialic acid using an inactive endosialidase-GFP fusion protein (I, II, III, IV)

5.4.1 Detection of polysialic acid in bacteria (I, IV)

The fusion protein with an inactive endosialidase was used to stain bacterial spots on glass slides. It was found to specifically recognize the polysialic acid-capsule of *E. coli* K1, but not the unrelated K2 capsule (I, Figure 5, panels A and B). The α2,8 polysialic acid-capsules of group B *Neisseria meningiditis* (I, Figure 5, panel G), *Mannheimia (Pasteurella) haemolytica* A2 (I, Figure 5, panels C and D) and *Moraxella nonliquefaciens* (I, Figure 5I) were also detected as well, but not the closely related strains that contain no polysialic acid capsule (I, Figure 5, panels E, F, H and J). It is noticeable that the fusion protein specifically bound to the α2,8 polysialic acid capsule of group B meningococci, but not to the related α2,9 polysialic acid capsule of group C meningococci (IV Table 1; I, Figure 5, panels G and H). Thus, the fusion protein efficiently differentiated α2,8 polysialic acid-positive and -negative strains.

The fusion protein is also adaptable to fluorometric microtiter plate assays. Microtiter plates coated with the polysialic acid containing K1 bacteria stained with the fusion protein emitted fluorescence, whereas K2 bacteria containing an unrelated capsule did not (I, Figure 6).

5.4.2 Detection of eukaryotic polysialic acid (I, II, IV)

The fusion protein with the inactivated endosialidase that specifically recognizes polysialic acid provides a powerful tool for the detection of this antigen in body fluids and histological specimens. The fusion protein specifically stained neuroblastoma SH-SY5Y cells (II, Figure 5) and neuroblastoma cells in paraffin-embedded sections (IV, Figure 7). It stained cells both in undifferentiated (I, Figure 7, panels A, D and E) and differentiated neuroblastoma specimens (I, Figure 7, panel F). Similar staining patterns were obtained for the monoclonal polysialic acid antibody 735 and the fusion protein (I, Figure 7, panels A and C). In addition the staining was specifically blocked by the addition of free polysialic acid (I, Figure 7, panel B).

In order to investigate the applicability of the fusion protein with the inactive endosialidase in the Western blot analysis, extracts of the rat brain homogenates were subjected to gel electroforesis and, after a transfer, the blots were stained with the fusion protein and visualized with a fluorescence reader. Only the fusion protein containing the catalytically inactive endosialise revealed a polysialic acid-containing band, corresponding to the mobility of the NCAM (I, Figure 4).

The embryonic polysialic acid-containing NCAM and the adult, non-polysialylated form of NCAM were also analyzed with a fluorescence microtiter plate assay using the fusion protein with the inactive endosialidase. The embryonic form of NCAM gave a clear fluorescence signal, whereas the adult, non-polysialylated form remained undetectable (I, Figure 6, panel B). The fusion protein also stained embryonal polysialic acid in fetal brains (II, Figure 6).

The fusion protein was also used for detecting neuroblastic cells in the bone marrow with flow cytometry and for investigating the role of polysialic acid as a molecular marker in neuroblastoma (Korja *et al.*, 2009). Patients whose neuroblastoma was positive for polysialic acid had metastases more often at the time of the diagnosis, and the expression of polysialylated NCAM was associated with an advanced disease state. Thus, polysialic acid could be used as a metastatic marker in neuroblastoma (Korja *et al.*, 2009), like in rhabdomyosarcoma, non-small cell lung carcinoma (NSCLC) and small cell lung carcinoma (SCLC) (Moolenaar *et al.*, 1990; Glüer *et al.*, 1998b; Tanaka *et al.*, 2000).

5.4.3 Expression of polysialic acid in an experimental *E. coli* K1 infection model (III)

The expression of polysialic acid in different phases of the neonatal *E. coli* K1 infection was studied in an experimental model. The human neonatal K1 infection begins with colonization of the intestine. The intestine of the newborn infant begins to evolve over the first few weeks of life and only after the first year of life do the microbiota develop into complex microbiota similar to those found in adults (Palmer *et al.*, 2007). The underdevelopment of the intestinal microbiota makes the colonization of harmful bacteria easier.

Neonatal immune responses to bacteria having a polysialic acid capsule, such as *E coli* K1, are weak; this is because the carbohydrate of the bacterial capsule is similar to polysialic acid in the host tissues. Progression of *E. coli* K1 infection was followed by counting viable bacteria from the tissue homogenates of the major organs and from the blood two and three days after feeding the rats with bacteria (III, Figure 1, panels A and B). The expression of the K1 capsule in the blood and tissue samples of the infected neonatal rats was studied with the fusion protein to specifically detect polysialic acid. Staining of blood sample smears of infected animals revealed that the majority of the *E. coli* bacteria in blood expressed polysialic acid (III, Figure 4). The polysialic acid capsule is probably needed for the protection of the bacteria from complement and opsonization.

Of different meningitis-causing bacteria only *E. coli* K1 invaded meningioma cells directly and induced rapid cell death before the inducement of an inflammatory response (Fowler *et al.*, 2004). Thus, the K1 polysialic acid capsule has been regarded as an invasion-promoting factor. The distribution of bacteria in the brain sagittal section was examined. The bacteria were restricted to the choroid plexus (III, Figure 2, panel A), cerebral folds (III, Figure 2, panels B and C) and meninges (III, Figure 2, panels D, E and F). Colonization of *E. coli* to the choroid plexus enables them to pass into the cerebrospinal fluid (CSF) and the ventricles. Therefore, polysialic acid-expressing bacterial cells in the ventricles were inspected. It was found that the bacteria expressed polysialic acid on the surface of the ependymal cells lining the ventricle (III, Figure 6). When *E. coli* K1 reached the meninges the bacteria ceased to express polysialic acid; however, bacteria expressing the O18 antigen could be detected (III, Figure 7). This was a new and unexpected result. The ending of the polysialic acid expression was not due to inhibition of the extensive *O*-acetylation of capsule, because the bacteria could not be detected with the *O*-acetyl-polysialic acid antibody. In order to test wheather the K1 bacteria able to invade the brain tissue had lost the capacity to synthesize polysialic acid, bacteria from the brain and other tissues were cultured and tested for their susceptibility to K1-specific bacteriophages. All bacteria cultured from different organs were found to be susceptible to the K1 phage and were thus capable of polysialic acid synthesis.

5.5 Future perspectives

The poor immunogenicity of polysialic acid has long restricted the production of reagents to study polysialic acid and its use for diagnostic purposes. Both monoclonal and polyclonal antibodies have been reported, especially in the search for vaccines against the group B meningococci. There is, however, only a limited number of reagents suitable for detection and identification of polysialic acid-containing bacteria or cells. The use of the inactivated enzyme has some advantages in comparison to antibodies. Its production is simple and reproducible with recombinant techniques, there are no cross-reactions with antibody-binding proteins of tissues and there is no need to use animals for its production. Furthermore, the use of the fusion protein provides a single-step staining procedure as opposed to the antibodies that generally involve multiple steps.

The fusion protein shows high specificity for α2,8-linked polysialic acid. No reaction is seen with α2,9-linked polysialic acid. The only cross-reactivity was detected with the rare *E. coli* K92 capsule with alternating α2,8/α2,9-linkages. Both active and inactive phages are known to react with this polysialic acid (Kwiatkowski *et al.*, 1982; Aalto *et al.*, 2001), and recent results indicate that the fusion protein also reacts with these bacteria. However, polysialic acid with

alternating α2,8/α2,9-linkages have not been found in mammals and, therefore, the fusion protein may be used as a marker for α2,8 polysialic acid in mammalian cells and tissues.

The structural model of the PK1A endosialidase reveals that both amino acid substitutions responsible for the inactivation of the enzyme localize near to its putative active site. Neither of the substitutions alone was able to inactivate the enzyme. However, conclusions on, for example, the binding mode of polysialic acid can only be drawn after the three-dimensional structure of the PK1A endosialidase has been solved. Attempts to produce diffracting crystals of the enzyme have not yet been successful.

In this study, we have described a new type of antibody substitute that utilizes the specificity of an enzyme to recognize its substrate. Potentially, inactivated enzymes represent a new way to develop specific detection reagents. Enzyme–substrate interactions are comparable to lectin–carbohydrate interactions, which are widely used as molecular tools. According to the definition, lectins are carbohydrate-binding proteins which have no catalytic activity. Enzymes, such as glycosidases and glycosyltransferases, are carbohydrate-binding molecules, but they are not defined as lectins due to their substrate cleaving activity. Throught deliberate inactivation of the enzymic activity without change to the binding properties, enzymes acting on carbohydrates could be converted to lectins. More generally, enzymes acting on different classes of macromolecules could be converted to molecules specifically binding to them without exercising their enzymic activity. In nucleotide and protein databases, a significant portion of all sequences belong to enzymes. Tens of thousands of three-dimensional structures have been solved, and databases on the active sites of enzymes have been created (Porter *et al.*, 2004). Thus, the number of enzyme–substrate interactions characterized in detail is enormous. These data offer a basis which can be exploited in the development of new molecular tools with predicted specificities.

6 SUMMARY

The present study focuses on designing, characterizing and testing a fusion protein based on inactive endosialidase for the detection of polysialic acid. In addition, the molecular background of the inactivation was investigated. On the basis of the results obtained, the following conclusions can be made:

1. A fusion protein, containing the inactive endosialidase and GFP, designed in this work proved to be a specific and effective one-step reagent for polysialic acid detection.

2. The use of the inactive endosialidase for the detection of polysialic acid was based on its ability to bind but not cleave polysialic acid.

3. The molecular basis of the inactivation of the endosialidase is due to two point mutations, which both are needed for the inactivation. Both of these mutations are located near the active site of the enzyme.

4. The fusion protein can be applied to the detection of both eukaryotic and prokaryotic polysialic acid irrespective of species, and used in fluorescent microscopy, western blots and fluorescent counting.

ACKNOWLEDGEMENTS

This study was carried out at the Department of Medical Biochemistry and Genetics, University of Turku. I would like to thank Professors *Klaus Elenius, Marko Salmi, Eero Vuorio, Anna-Marja Säämänen, Jukka Finne* and Prof. emeritus *Risto Penttinen* for providing excellent working facilities. I also thank Professor *Hannu Järveläinen* for his interest in my work.

I owe my sincere gratitude to my supervisor Professor *Jukka Finne*. I particularly respect his knowledge, ideas and the way to revise manuscripts precisely and carefully. He has also given me plenty of freedom in this work, which I appreciate.

The Glycoscience Graduate School is acknowledged for educational support as well as unforgettable moments during the spare time of meetings. I also thank my supervisory committee Professor *Veijo Hukkanen* and Docent *Markku Kallajoki.*

I acknowledge Docent *Anne Leppänen* and Professor *Mikael Skurnik* for reviewing the thesis manuscript and invaluable comments. I am also grateful to *Maaria* and *Damon Tringham* for revising the language of the thesis manuscript.

I wish to thank Professor *Peter Taylor* for fruitful collaboration. I also warmly thank my other co-authors Drs *Juha Aalto, Mark Bowen, Sauli Haataja, Harri Hirvonen, Elina Jakobsson, Miikka Korja, Jukka Lehtonen, Vuokko Loimaranta, Paul Luzio, Pauli Ollikka* and *Andrea Zelmer* for their contribution. I am also grateful to *Jukka Lehtonen* for magnificent structural figures.

I thank all the present and former members of Professor Finne's research group for their support and friendship. *Miikka*, thank you for the collaboration during all these years, your contribution is invaluable for this work. *Elina*, I have had the pleasure to work with you for a long time, thank you for these years both in and outside the laboratory. *Pauli*, your good advice and help were very valuable for this work. *Vuokko*, I thank you for the moments in sport activities and your support. *Annika*, thank you for your help and support. *Sauli,* thank you for discussions and your interest in my work. *Elise Pinta*, from our neighbouring group, thank you for fruitful discussions and company.

I want to thank *Erika Ekholm* for friendship and collaboration, *Johanna Holmbom* for friendship starting from "cola breaks", *Anna Karhu* for excellent work in my projects, *Saija Kiljunen* for help and encouragement, and *Heikki Peuravuori* for friendship.

Jukka Karhu, Terttu Jompero, Marja Nykänen, Liisa Peltonen, Raili Salonen as well as other technical personnel are acknowledged for excellent technical assistance during these years. I also wish to thank *Rakel Mattsson* for secretarial help and unofficial discussions.

In addition, I thank many other co-workers in our department and in the scientific community of Turku.

I am deeply grateful to my parents Kirsti and Ilmari, my sister Teija and brother Jouko, and their families, as well as all my friends for their support.

I wish to warmly thank Jussi for his love, support and encouragement.

This work has been financially supported by the Academy of Finland, the Medical Faculty of the Turku University, the Turku University Foundation, the Glycoscience Graduate School and the Magnus Ehrnrooth Foundation.

Turku, January 2010

Anne Jokilammi

REFERENCES

Aalto, J., Pelkonen, S., Kalimo, H. and Finne, J. (2001) Mutant bacteriophage with non-catalytic endosialidase binds to both bacterial and eukaryotic polysialic acid and can be used as probe for its detection. Glycoconj.J. 18, 751.

Acheson, A., Sunshine, J.L. and Rutishauser, U. (1991) NCAM polysialic acid can regulate both cell-cell and cell-substrate interactions. J.Cell Biol. 114, 143.

Achtman, M., Mercer, A., Kusecek, B., Pohl, A., Heuzenroeder, M., Aaronson, W., Sutton, A. and Silver, R.P. (1983) Six widespread bacterial clones among Escherichia coli K1 isolates. Infect.Immun. 39, 315.

Ackermann, H.W. (2003) Bacteriophage observations and evolution. Res.Microbiol. 154, 245.

Ackermann, H.W. (1998) Tailed bacteriophages: the order caudovirales. Adv.Virus Res. 51, 135.

Allen, P.Z., Glode, M., Schneerson, R. and Robbins, J.B. (1982) Identification of immunoglobulin heavy-chain isotypes of specific antibodies of horse 46 group B meningococcal antiserum. J.Clin.Microbiol. 15, 324.

Angata, K. and Fukuda, M. (2003) Polysialyltransferases: major players in polysialic acid synthesis on the neural cell adhesion molecule. Biochimie 85, 195.

Azurmendi, H.F., Vionnet, J., Wrightson, L., Trinh, L.B., Shiloach, J. and Freedberg, D.I. (2007) Extracellular structure of polysialic acid explored by on cell solution NMR. Proc.Natl.Acad.Sci.U.S.A. 104, 11557.

Barbeau, D., Liang, J.J., Robitalille, Y., Quirion, R. and Srivastava, L.K. (1995) Decreased expression of the embryonic form of the neural cell adhesion molecule in schizophrenic brains. Proc.Natl.Acad.Sci.U.S.A. 92, 2785.

Barry, G.T. (1958) Colominic acid, a polymer of N-acetylneuraminic acid. J.Exp.Med. 107, 507.

Barry, G.T. and Goebel, W.F. (1957) Colominic acid, a substance of bacterial origin related to sialic acid. Nature 179, 206.

Bayer, M.E., Thurow, H. and Bayer, M.H. (1979) Penetration of the polysaccharide capsule of Escherichia coli (Bi161/42) by bacteriophage K29. Virology 94, 95.

Berski, S., van Bergeijk, J., Schwarzer, D., Stark, Y., Kasper, C., Scheper, T., Grothe, C., Gerardy-Schahn, R., Kirschning, A. and Dräger, G. (2008) Synthesis and biological evaluation of a polysialic acid-based hydrogel as enzymatically degradable scaffold material for tissue engineering. Biomacromolecules 9, 2353.

Bhattacharjee, A.K., Jennings, H.J., Kenny, C.P., Martin, A. and Smith, I.C. (1976) Structural determination of the polysaccharide antigens of Neisseria meningitidis serogroups Y, W-135, and BO1. Can.J.Biochem. 54, 1.

Bonfanti, L. (2006) PSA-NCAM in mammalian structural plasticity and neurogenesis. Prog.Neurobiol. 80, 129.

Bøvre, K., Bryn, K., Closs, O., Hagen, N. and Froholm, L.O. (1983) Surface polysaccharide of Moraxella non-liquefaciens identical to Neisseria meningitidis group B capsular polysaccharide. A chemical and immunological investigation. NIPH Ann. 6, 65.

Brandi L. Cantarel, Pedro M., C., Corinne Rancuel, Thomas Bernard and Vincent Lombard and Bernard Henrissant. (2009) The Carbohydrate-Active EnZymes database (CAZy): an expert resource for Glycogenomics. Nucleic Acids Research 37, D233.

Brusés, J.L. and Rutishauser, U. (2001) Roles, regulation, and mechanism of polysialic acid function during neural development. Biochimie 83, 635.

Cheng, M.C., Lin, S.L., Wu, S.H., Inoue, S. and Inoue, Y. (1998) High-performance capillary electrophoretic characterization of different types of oligo- and polysialic acid chains. Anal.Biochem. 260, 154.

Cheung, I.Y., Vickers, A. and Cheung, N.K. (2006) Sialyltransferase STX (ST8SiaII): a novel molecular marker of metastatic neuroblastoma. Int.J.Cancer 119, 152.

Chien, C.H., Shann, Y.J. and Sheu, S.Y. (1996) Site-directed mutations of the catalytic and conserved amino acids of the neuraminidase gene, nanH, of Clostridium perfringens ATCC 10543. Enzyme Microb.Technol. 19, 267.

Constantinou, A., Epenetos, A.A., Hreczuk-Hirst, D., Jain, S. and Deonarain, M.P. (2008) Modulation of antibody pharmacokinetics by chemical polysialylation. Bioconjug.Chem. 19, 643.

Constantinou, A., Epenetos, A.A., Hreczuk-Hirst, D., Jain, S., Wright, M., Chester, K.A. and Deonarain, M.P. (2009) Site-Specific Polysialylation of an Antitumor Single-Chain Fv Fragment. Bioconjug.Chem. 20, 924.

Cremer, H., Lange, R., Christoph, A., Plomann, M., Vopper, G., Roes, J., Brown, R., Baldwin, S., Kraemer, P. and Scheff, S. (1994) Inactivation of the N-CAM gene in mice results in size reduction of the olfactory bulb and deficits in spatial learning. Nature 367, 455.

Crennell, S., Garman, E., Laver, G., Vimr, E. and Taylor, G. (1994) Crystal structure of Vibrio cholerae neuraminidase reveals dual lectin-like domains in addition to the catalytic domain. Structure 2, 535.

Cross, A.S., Kim, K.S., Wright, D.C., Sadoff, J.C. and Gemski, P. (1986) Role of lipopolysaccharide and capsule in the serum resistance of bacteremic strains of Escherichia coli. J.Infect.Dis. 154, 497.

Cross, M.A., Koronakis, V., Stanley, P.L. and Hughes, C. (1990) HlyB-dependent secretion of hemolysin by uropathogenic Escherichia coli requires conserved sequences flanking the chromosomal hly determinant. J.Bacteriol. 172, 1217.

Deszo, E.L., Steenbergen, S.M., Freedberg, D.I. and Vimr, E.R. (2005) Escherichia coli K1 polysialic acid O-acetyltransferase gene, neuO, and the mechanism of capsule form variation involving a mobile contingency locus. Proc.Natl.Acad.Sci.U.S.A. 102, 5564.

Dodd, J., Morton, S.B., Karagogeos, D., Yamamoto, M. and Jessell, T.M. (1988) Spatial regulation of axonal glycoprotein expression on subsets of embryonic spinal neurons. Neuron 1, 105.

Doyle, E., Nolan, P.M., Bell, R. and Regan, C.M. (1992) Hippocampal NCAM180 transiently increases sialylation during the acquisition and consolidation of a passive avoidance response in the adult rat. J.Neurosci.Res. 31, 513.

Drake, P.M., Nathan, J.K., Stock, C.M., Chang, P.V., Muench, M.O., Nakata, D., Reader, J.R., Gip, P., Golden, K.P., Weinhold, B., Gerardy-Schahn, R., Troy, F.A.,2nd and Bertozzi, C.R. (2008) Polysialic acid, a glycan with highly restricted expression, is found on human and murine leukocytes and modulates immune responses. J.Immunol. 181, 6850.

Dubois, C., Figarella-Branger, D., Pastoret, C., Rampini, C., Karpati, G. and Rougon, G. (1994) Expression of NCAM and its polysialylated isoforms during mdx mouse muscle regeneration and in vitro myogenesis. Neuromuscul.Disord. 4, 171.

El Maarouf, A., Petridis, A.K. and Rutishauser, U. (2006) Use of polysialic acid in repair of the central nervous system. Proc.Natl.Acad.Sci.U.S.A. 103, 16989.

Fernandes, A.I. and Gregoriadis, G. (1997) Polysialylated asparaginase: preparation, activity and pharmacokinetics. Biochim.Biophys.Acta 1341, 26.

Figarella-Branger, D., Dubois, C., Chauvin, P., De Victor, B., Gentet, J.C. and Rougon, G. (1996) Correlation between polysialic-neural cell adhesion molecule levels in CSF and medulloblastoma outcomes. J.Clin.Oncol. 14, 2066.

Finne, J. (1982) Occurrence of unique polysialosyl carbohydrate units in glycoproteins of developing brain. J.Biol.Chem. 257, 11966.

Finne, J., Bitter-Suermann, D., Goridis, C. and Finne, U. (1987) An IgG monoclonal antibody to group B meningococci cross-reacts with developmentally regulated polysialic acid units of glycoproteins in neural and extraneural tissues. J.Immunol. 138, 4402.

Finne, J., Finne, U., Deagostini-Bazin, H. and Goridis, C. (1983) Occurrence of alpha 2-8 linked polysialosyl units in a neural cell adhesion molecule. Biochem.Biophys.Res.Commun. 112, 482.

Finne, J., Krusius, T., Rauvala, H. and Hemminki, K. (1977) The disialosyl group of glycoproteins. Occurrence in different tissues and cellular membranes. Eur.J.Biochem. 77, 319.

Finne, J., Leinonen, M. and Mäkelä, P.H. (1983) Antigenic similarities between brain components and bacteria causing meningitis. Implications for vaccine development and pathogenesis. Lancet 2, 355.

Finne, J. and Mäkelä, P.H. (1985) Cleavage of the polysialosyl units of brain glycoproteins by a bacteriophage endosialidase. Involvement of a long oligosaccharide segment in molecular interactions of polysialic acid. J.Biol.Chem. 260, 1265.

Fowler, M.I., Weller, R.O., Heckels, J.E. and Christodoulides, M. (2004) Different meningitis-causing bacteria induce distinct inflammatory responses on interaction with cells of the human meninges. Cell.Microbiol. 6, 555.

Frosch, M., Görgen, I., Boulnois, G.J., Timmis, K.N. and Bitter-Suermann, D. (1985) NZB mouse system for production of monoclonal antibodies to weak bacterial antigens: isolation of an IgG antibody to the polysaccharide capsules of Escherichia coli K1 and group B meningococci. Proc.Natl.Acad.Sci.U.S.A. 82, 1194.

Fujimoto, I., Bruses, J.L. and Rutishauser, U. (2001) Regulation of cell adhesion by polysialic acid. Effects on cadherin, immunoglobulin cell adhesion molecule, and integrin function and independence from neural cell adhesion molecule binding or signaling activity. J.Biol.Chem. 276, 31745.

Galuska, S.P., Geyer, R., Mühlenhoff, M. and Geyer, H. (2007) Characterization of oligo- and polysialic acids by MALDI-TOF-MS. Anal.Chem. 79, 7161.

Gascon, E., Vutskits, L. and Kiss, J.Z. (2008) The Role of PSA-NCAM in Adult Neurogenesis. Neurochem.Res. doi:10.1007/s11064-008-9744-3.

Gaskell, A., Crennell, S. and Taylor, G. (1995) The three domains of a bacterial sialidase: a beta-propeller, an immunoglobulin module and a galactose-binding jelly-roll. Structure 3, 1197.

Gerardy-Schahn, R., Bethe, A., Brennecke, T., Mühlenhoff, M., Eckhardt, M., Ziesing, S., Lottspeich, F. and Frosch, M. (1995) Molecular cloning and functional expression of bacteriophage PK1E-encoded endoneuraminidase Endo NE. Mol.Microbiol. 16, 441.

Glick, M.C., Livingston, B.D., Shaw, G.W., Jacobs, J.L. and Troy, F.A. (1991) Expression of polysialic acid on human neuroblastoma. Prog.Clin.Biol.Res. 366, 267.

Glode, M.P., Sutton, A., Moxon, E.R. and Robbins, J.B. (1977) Pathogenesis of neonatal Escherichia coli meningitis: induction of bacteremia and meningitis in infant rats fed E. coli K1. Infect.Immun. 16, 75.

Glüer, S., Schelp, C., Madry, N., von Schweinitz, D., Eckhardt, M. and Gerardy-Schahn, R. (1998a) Serum polysialylated neural cell adhesion molecule in childhood neuroblastoma. Br.J.Cancer 78, 106.

Glüer, S., Schelp, C., von Schweinitz, D. and Gerardy-Schahn, R. (1998b) Polysialylated neural cell adhesion molecule in childhood rhabdomyosarcoma. Pediatr.Res. 43, 145.

Gregoriadis, G., Jain, S., Papaioannou, I. and Laing, P. (2005) Improving the therapeutic efficacy of peptides and proteins: a role for polysialic acids. Int.J.Pharm. 300, 125.

Gross, R.J., Cheasty, T. and Rowe, B. (1977) Isolation of bacteriophages specific for the K1 polysaccharide antigen of Escherichia coli. J.Clin.Microbiol. 6, 548.

Haile, Y., Berski, S., Dräger, G., Nobre, A., Stummeyer, K., Gerardy-Schahn, R. and Grothe, C. (2008) The effect of modified polysialic acid based hydrogels on the adhesion and viability of primary neurons and glial cells. Biomaterials 29, 1880.

Haile, Y., Haastert, K., Cesnulevicius, K., Stummeyer, K., Timmer, M., Berski, S., Dräger, G., Gerardy-Schahn, R. and Grothe, C. (2007) Culturing of glial and neuronal cells on polysialic acid. Biomaterials 28, 1163.

Halberstadt, J.B., Flowers, H. and Glick, M.C. (1993) A method to detect polysialic acid in polymers of 10 or more sialyl residues synthesized in vivo and in vitro. Anal.Biochem. 209, 136.

Hallenbeck, P.C., Vimr, E.R., Yu, F., Bassler, B. and Troy, F.A. (1987a) Purification and properties of a bacteriophage-induced endo-N-acetylneuraminidase specific for poly-alpha-2,8-sialosyl carbohydrate units. J.Biol.Chem. 262, 3553.

Hallenbeck, P.C., Yu, F. and Troy, F.A. (1987b) Rapid separation of oligomers of polysialic acid by high-performance liquid chromatography. Anal.Biochem. 161, 181.

Harris, J.M. and Chess, R.B. (2003) Effect of pegylation on pharmaceuticals. Nat.Rev.Drug Discov. 2, 214.

Häyrinen, J., Haseley, S., Talaga, P., Mühlenhoff, M., Finne, J. and Vliegenthart, J.F. (2002) High affinity binding of long-

chain polysialic acid to antibody, and modulation by divalent cations and polyamines. Mol.Immunol. 39, 399.

Hildebrandt, H., Becker, C., Glüer, S., Rösner, H., Gerardy-Schahn, R. and Rahmann, H. (1998) Polysialic acid on the neural cell adhesion molecule correlates with expression of polysialyltransferases and promotes neuroblastoma cell growth. Cancer Res. 58, 779.

Hoiczyk, E., Roggenkamp, A., Reichenbecher, M., Lupas, A. and Heesemann, J. (2000) Structure and sequence analysis of Yersinia YadA and Moraxella UspAs reveal a novel class of adhesins. EMBO J. 19, 5989.

Horn, S.J., Sikorski, P., Cederkvist, J.B., Vaaje-Kolstad, G., Sørlie, M., Synstad, B., Vriend, G., Vårum, K.M. and Eijsink, V.G. (2006) Costs and benefits of processivity in enzymatic degradation of recalcitrant polysaccharides. Proc.Natl.Acad.Sci.U.S.A. 103, 18089.

Husmann, M., Roth, J., Kabat, E.A., Weisgerber, C., Frosch, M. and Bitter-Suermann, D. (1990) Immunohistochemical localization of polysialic acid in tissue sections: differential binding to polynucleotides and DNA of a murine IgG and a human IgM monoclonal antibody. J.Histochem.Cytochem. 38, 209.

Inoue, S. and Inoue, Y. (2003) Ultrasensitive analysis of sialic acids and oligo/polysialic acids by fluorometric high-performance liquid chromatography. Methods Enzymol. 362, 543.

Inoue, S. and Iwasaki, M. (1978) Isolation of a novel glycoprotein from the eggs of rainbow trout: occurrence of disialosyl groups on all carbohydrate chains. Biochem.Biophys.Res.Commun. 83, 1018.

Inoue, S., Lin, S.L. and Inoue, Y. (2000) Chemical analysis of the developmental pattern of polysialylation in chicken brain. Expression of only an extended form of polysialyl chains during embryogenesis and the presence of disialyl residues in both embryonic and adult chicken brains. J.Biol.Chem. 275, 29968.

Inoue, S., Lin, S.L., Inoue, Y., Groves, D.R., Thomson, R.J., von Itzstein, M., Pavlova, N.V., Li, S.C. and Li, Y.T. (2001a) A unique sialidase that cleaves the Neu5Gcalpha2–>5-O(glycolyl)Neu5Gc linkage: comparison of its specificity with that of three microbial sialidases toward four sialic acid dimers. Biochem.Biophys.Res.Commun. 280, 104.

Inoue, S., Lin, S.L., Lee, Y.C. and Inoue, Y. (2001b) An ultrasensitive chemical method for polysialic acid analysis. Glycobiology 11, 759.

Jain, S., Hreczuk-Hirst, D.H., McCormack, B., Mital, M., Epenetos, A., Laing, P. and Gregoriadis, G. (2003) Polysialylated insulin: synthesis, characterization and biological activity in vivo. Biochim.Biophys.Acta 1622, 42.

James, W.M. and Agnew, W.S. (1987) Multiple oligosaccharide chains in the voltage-sensitive Na channel from electrophorus electricus: evidence for alpha-2,8-linked polysialic acid. Biochem.Biophys.Res.Commun. 148, 817.

Jennings, H.J., Katzennellenbogen, E., Lugowski, C., Michon, F., Roy, R. and Kasper, D.L. (1984) Structure, conformation, and immunology of sialic acid-containing polysaccharides of human pathogenic bacteria. Pure Appl Chem 56, 893.

Johnson, C.P., Fragneto, G., Konovalov, O., Dubosclard, V., Legrand, J.F. and Leckband, D.E. (2005a) Structural studies of the neural-cell-adhesion molecule by X-ray and neutron reflectivity. Biochemistry 44, 546.

Johnson, C.P., Fujimoto, I., Rutishauser, U. and Leckband, D.E. (2005b) Direct evidence that neural cell adhesion molecule (NCAM) polysialylation increases intermembrane repulsion and abrogates adhesion. J.Biol.Chem. 280, 137.

Johnson, M. S. and Overington, J. P. (1993) A structural basis for sequence comparisons. An evaluation of scoring methodologies. J. Mol. Biol. 233, 716–738.

Kabat, E.A., Liao, J., Osserman, E.F., Gamian, A., Michon, F. and Jennings, H.J. (1988) The epitope associated with the binding of the capsular polysaccharide of the group B meningococcus and of Escherichia coli K1 to a human monoclonal macroglobulin, IgMNOV. J.Exp.Med. 168, 699.

Kabat, E.A., Nickerson, K.G., Liao, J., Grossbard, L., Osserman, E.F., Glickman, E., Chess, L., Robbins, J.B., Schneerson, R. and Yang, Y.H. (1986) A human monoclonal macroglobulin with specificity for alpha(2–>8)-linked poly-N-acetyl

neuraminic acid, the capsular polysaccharide of group B meningococci and Escherichia coli K1, which crossreacts with polynucleotides and with denatured DNA. J.Exp.Med. 164, 642.

Kaper, J.B., Nataro, J.P. and Mobley, H.L. (2004) Pathogenic Escherichia coli. Nat.Rev.Microbiol. 2, 123.

Kataoka, Y., Miyake, K. and Iijima, S. (2006) Coliphage derived sialidase preferentially recognizes nonreducing end of polysialic acid. J.Biosci.Bioeng. 101, 198.

Kern, W.F., Spier, C.M., Hanneman, E.H., Miller, T.P., Matzner, M. and Grogan, T.M. (1992) Neural cell adhesion molecule-positive peripheral T-cell lymphoma: a rare variant with a propensity for unusual sites of involvement. Blood 79, 2432.

Kibbelaar, R.E., Moolenaar, C.E., Michalides, R.J., Bitter-Suermann, D., Addis, B.J. and Mooi, W.J. (1989) Expression of the embryonal neural cell adhesion molecule N-CAM in lung carcinoma. Diagnostic usefulness of monoclonal antibody 735 for the distinction between small cell lung cancer and non-small cell lung cancer. J.Pathol. 159, 23.

Kibbelaar, R.E., Moolenaar, K.E., Michalides, R.J., Van Bodegom, P.C., Vanderschueren, R.G., Wagenaar, S.S., Dingemans, K.P., Bitter-Suermann, D., Dalesio, O. and Van Zandwijk, N. (1991) Neural cell adhesion molecule expression, neuroendocrine differentiation and prognosis in lung carcinoma. Eur.J.Cancer 27, 431.

Kim, K.J., Elliott, S.J., Di Cello, F., Stins, M.F. and Kim, K.S. (2003) The K1 capsule modulates trafficking of E. coli-containing vacuoles and enhances intracellular bacterial survival in human brain microvascular endothelial cells. Cell.Microbiol. 5, 245.

Kiss, J.Z. and Rougon, G. (1997) Cell biology of polysialic acid. Curr.Opin.Neurobiol. 7, 640.

Kitazume, S., Kitajima, K., Inoue, S. and Inoue, Y. (1992) Detection, isolation, and characterization of oligo/poly(sialic acid) and oligo/poly(deaminoneuraminic acid) units in glycoconjugates. Anal.Biochem. 202, 25.

Korhonen, T.K., Valtonen, M.V., Parkkinen, J., Väisänen-Rhen, V., Finne, J., Ørskov, F., Ørskov, I., Svenson, S.B. and Mäkelä, P.H. (1985) Serotypes, hemolysin production, and receptor recognition of Escherichia coli strains associated with neonatal sepsis and meningitis. Infect.Immun. 48, 486.

Korja, M., Jokilammi, A., Salmi, T.T., Kalimo, H., Pelliniemi, T.T., Isola, J., Rantala, I., Haapasalo, H. and Finne, J. (2009) Absence of polysialylated NCAM is an unfavorable prognostic phenotype for advanced stage neuroblastoma. BMC Cancer 9, 57.

Kwiatkowski, B., Boschek, B., Thiele, H. and Stirm, S. (1983) Substrate specificity of two bacteriophage-associated endo-N-acetylneuraminidases. J.Virol. 45, 367.

Kwiatkowski, B., Boschek, B., Thiele, H. and Stirm, S. (1982) Endo-N-acetylneuraminidase associated with bacteriophage particles. J.Virol. 43, 697.

Lackie, P.M., Zuber, C. and Roth, J. (1991) Expression of polysialylated N-CAM during rat heart development. Differentiation 47, 85.

Langer, R. and Vacanti, J.P. (1993) Tissue engineering. Science 260, 920.

Leggate, D.R., Bryant, J.M., Redpath, M.B., Head, D., Taylor, P.W. and Luzio, J.P. (2002) Expression, mutagenesis and kinetic analysis of recombinant K1E endosialidase to define the site of proteolytic processing and requirements for catalysis. Mol.Microbiol. 44, 749.

Lehtonen, J. V., Still, D.-J., Rantanen, V.-V., Ekholm, J., Björklund, D., Iftikhar, Z., Huhtala, M., Repo, S., Jussila, A., Jaakkola, J., Pentikäinen, O., Nyrönen, T., Salminen, T., Gyllenberg, M. and Johnson, M. S. (2004) BODIL: a molecular modeling environment for structure-function analysis and drug design. J. Comput. Aided Mol. Des. 18, 401–419

Leiman, P.G., Battisti, A.J., Bowman, V.D., Stummeyer, K., Mühlenhoff, M., Gerardy-Schahn, R., Scholl, D. and Molineux, I.J. (2007) The structures of bacteriophages K1E and K1-5 explain processive degradation of polysaccharide capsules and evolution of new host specificities. J.Mol.Biol. 371, 836.

Leying, H., Suerbaum, S., Kroll, H.P., Stahl, D. and Opferkuch, W. (1990) The capsular polysaccharide is a major determinant of serum resistance in K-1-positive blood culture isolates of Escherichia coli. Infect.Immun. 58, 222.

Lifely, M.R., Gilbert, A.S. and Moreno, C. (1981) Sialic acid polysaccharide antigens

of Neisseria meningitidis and Escherichia coli: esterification between adjacent residues. Carbohydr.Res. 94, 193.

Lipinski, M., Hirsch, M.R., Deagostini-Bazin, H., Yamada, O., Tursz, T. and Goridis, C. (1987) Characterization of neural cell adhesion molecules (NCAM) expressed by Ewing and neuroblastoma cell lines. Int.J.Cancer 40, 81.

Liu, T.Y., Gotschlich, E.C., Dunne, F.T. and Jonssen, E.K. (1971) Studies on the meningococcal polysaccharides. II. Composition and chemical properties of the group B and group C polysaccharide. J.Biol.Chem. 246, 4703.

Livingston, B.D., Jacobs, J.L., Glick, M.C. and Troy, F.A. (1988) Extended polysialic acid chains (n greater than 55) in glycoproteins from human neuroblastoma cells. J.Biol.Chem. 263, 9443.

Long, G.S., Bryant, J.M., Taylor, P.W. and Luzio, J.P. (1995) Complete nucleotide sequence of the gene encoding bacteriophage E endosialidase: implications for K1E endosialidase structure and function. Biochem.J. 309 (Pt 2), 543.

MacKenzie, C.R., Hirama, T., Deng, S.J., Bundle, D.R., Narang, S.A. and Young, N.M. (1996) Analysis by surface plasmon resonance of the influence of valence on the ligand binding affinity and kinetics of an anti-carbohydrate antibody. J.Biol.Chem. 271, 1527.

Mandrell, R.E. and Zollinger, W.D. (1982) Measurement of antibodies to meningococcal group B polysaccharide: low avidity binding and equilibrium binding constants. J.Immunol. 129, 2172.

Martersteck, C.M., Kedersha, N.L., Drapp, D.A., Tsui, T.G. and Colley, K.J. (1996) Unique alpha 2, 8-polysialylated glycoproteins in breast cancer and leukemia cells. Glycobiology 6, 289.

Mechtersheimer, G., Staudter, M. and Möller, P. (1991) Expression of the natural killer cell-associated antigens CD56 and CD57 in human neural and striated muscle cells and in their tumors. Cancer Res. 51, 1300.

Michon, F., Brisson, J.R. and Jennings, H.J. (1987) Conformational differences between linear alpha (2–>8)-linked homosialooligosaccharides and the epitope of the group B meningococcal polysaccharide. Biochemistry 26, 8399.

Mikkonen, M., Soininen, H., Kälviänen, R., Tapiola, T., Ylinen, A., Vapalahti, M., Paljärvi, L. and Pitkänen, A. (1998) Remodeling of neuronal circuitries in human temporal lobe epilepsy: increased expression of highly polysialylated neural cell adhesion molecule in the hippocampus and the entorhinal cortex. Ann.Neurol. 44, 923.

Mikkonen, M., Soininen, H., Tapiola, T., Alafuzoff, I. and Miettinen, R. (1999) Hippocampal plasticity in Alzheimer's disease: changes in highly polysialylated NCAM immunoreactivity in the hippocampal formation. Eur.J.Neurosci. 11, 1754.

Moolenaar, C.E., Muller, E.J., Schol, D.J., Figdor, C.G., Bock, E., Bitter-Suermann, D. and Michalides, R.J. (1990) Expression of neural cell adhesion molecule-related sialoglycoprotein in small cell lung cancer and neuroblastoma cell lines H69 and CHP-212. Cancer Res. 50, 1102.

Morley, T.J., Willis, L.M., Whitfield, C., Wakarchuk, W.W. and Withers, S.G. (2009) A new sialidase mechanism: bacteriophage K1F endo-sialidase is an inverting glycosidase. J.Biol.Chem. 284, 17404.

Moxon, E.R. and Kroll, J.S. (1990) The role of bacterial polysaccharide capsules as virulence factors. Curr.Top.Microbiol.Immunol. 150, 65.

Mühlenhoff, M., Eckhardt, M. and Gerardy-Schahn, R. (1998) Polysialic acid: three-dimensional structure, biosynthesis and function. Curr.Opin.Struct.Biol. 8, 558.

Mühlenhoff, M., Stummeyer, K., Grove, M., Sauerborn, M. and Gerardy-Schahn, R. (2003) Proteolytic processing and oligomerization of bacteriophage-derived endosialidases. J.Biol.Chem. 278, 12634.

Muller, D., Wang, C., Skibo, G., Toni, N., Cremer, H., Calaora, V., Rougon, G. and Kiss, J.Z. (1996) PSA-NCAM is required for activity-induced synaptic plasticity. Neuron 17, 413.

Murakami, S., Seki, T., Rutishauser, U. and Arai, Y. (2000) Enzymatic removal of polysialic acid from neural cell adhesion molecule perturbs the migration route of luteinizing hormone-releasing hormone neurons in the developing chick forebrain. J.Comp.Neurol. 420, 171.

Mushtaq, N., Redpath, M.B., Luzio, J.P. and Taylor, P.W. (2004) Prevention and cure of systemic Escherichia coli K1 infection by

modification of the bacterial phenotype. Antimicrob.Agents Chemother. 48, 1503.

Nakata, D. and Troy, F.A.,2nd. (2005) Degree of polymerization (DP) of polysialic acid (polySia) on neural cell adhesion molecules (N-CAMS): development and application of a new strategy to accurately determine the DP of polySia chains on N-CAMS. J.Biol.Chem. 280, 38305.

Nummelin, H., El Tahir, Y., Ollikka, P., Skurnik, M. and Goldman, A. (2002) Expression, purification and crystallization of a collagen-binding fragment of Yersinia adhesin YadA. Acta Crystallogr.D Biol.Crystallogr. 58, 1042.

Ong, E., Nakayama, J., Angata, K., Reyes, L., Katsuyama, T., Arai, Y. and Fukuda, M. (1998) Developmental regulation of polysialic acid synthesis in mouse directed by two polysialyltransferases, PST and STX. Glycobiology 8, 415.

Palmer, C., Bik, E.M., DiGiulio, D.B., Relman, D.A. and Brown, P.O. (2007) Development of the human infant intestinal microbiota. PLoS Biol. 5, e177. doi:10.1371/journal.pbio.0050177.

Pelkonen, S. (1990) Capsular sialyl chains of *Escherichia coli* K1 mutants resistant to K1 phage. Curr. Microbiol. 21, 23.

Pelkonen, S. and Finne, J. (1987) A rapid turbidimetric assay for the study of serum sensitivy of Escherchia coli. FEMS Microbiol. Lett. 42, 53-57.

Pelkonen, S., Aalto, J. and Finne, J. (1992) Differential activities of bacteriophage depolymerase on bacterial polysaccharide: binding is essential but degradation is inhibitory in phage infection of K1-defective Escherichia coli. J.Bacteriol. 174, 7757.

Pelkonen, S., Häyrinen, J. and Finne, J. (1988) Polyacrylamide gel electrophoresis of the capsular polysaccharides of Escherichia coli K1 and other bacteria. J.Bacteriol. 170, 2646.

Pelkonen, S., Pelkonen, J. and Finne, J. (1989) Common cleavage pattern of polysialic acid by bacteriophage endosialidases of different properties and origins. J.Virol. 63, 4409.

Pellequer, J.L. and Van Regenmortel, M.H. (1993) Affinity of monoclonal antibodies to large multivalent antigens: influence of steric hindrance on antibody affinity constants calculated from Scatchard plots. Mol.Immunol. 30, 955.

Petridis, A.K., El-Maarouf, A. and Rutishauser, U. (2004) Polysialic acid regulates cell contact-dependent neuronal differentiation of progenitor cells from the subventricular zone. Dev.Dyn. 230, 675.

Petridis, A.K., Wedderkopp, H., Hugo, H.H. and Maximilian Mehdorn, H. (2009) Polysialic acid overexpression in malignant astrocytomas. Acta Neurochir.(Wien) 151, 601.

Petter, J.G. and Vimr, E.R. (1993) Complete nucleotide sequence of the bacteriophage K1F tail gene encoding endo-N-acylneuraminidase (endo-N) and comparison to an endo-N homolog in bacteriophage PK1E. J.Bacteriol. 175, 4354.

Pham, K., Nacher, J., Hof, P.R. and McEwen, B.S. (2003) Repeated restraint stress suppresses neurogenesis and induces biphasic PSA-NCAM expression in the adult rat dentate gyrus. Eur.J.Neurosci. 17, 879.

Pluschke, G., Mercer, A., Kusecek, B., Pohl, A. and Achtman, M. (1983) Induction of bacteremia in newborn rats by Escherichia coli K1 is correlated with only certain O (lipopolysaccharide) antigen types. Infect.Immun. 39, 599.

Porter, C.T., Bartlett, G.J. and Thornton, J.M. (2004) The Catalytic Site Atlas: a resource of catalytic sites and residues identified in enzymes using structural data. Nucleic Acids Res. 32 Database issue, D129.

Rauvala, H. and Kärkkäinen, J.ä. (1977) Methylation analysis of neuraminic acids by gas chromatography-mass spectrometry. Carbohydr.Res. 56, 1.

Robbins, J.B., McCracken, G.H.,Jr, Gotschlich, E.C., Ørskov, F., Ørskov, I. and Hanson, L.A. (1974) Escherichia coli K1 capsular polysaccharide associated with neonatal meningitis. N.Engl.J.Med. 290, 1216.

Rohr, T.E. and Troy, F.A. (1980) Structure and biosynthesis of surface polymers containing polysialic acid in Escherichia coli. J.Biol.Chem. 255, 2332.

Roth, J., Taatjes, D.J., Bitter-Suermann, D. and Finne, J. (1987) Polysialic acid units are spatially and temporally expressed in developing postnatal rat kidney. Proc.Natl.Acad.Sci.U.S.A. 84, 1969.

Roth, J., Zuber, C., Wagner, P., Taatjes, D.J., Weisgerber, C., Heitz, P.U., Goridis, C. and

Bitter-Suermann, D. (1988) Reexpression of poly(sialic acid) units of the neural cell adhesion molecule in Wilms tumor. Proc.Natl.Acad.Sci.U.S.A. 85, 2999.

Rougon, G., Dubois, C., Buckley, N., Magnani, J.L. and Zollinger, W. (1986) A monoclonal antibody against meningococcus group B polysaccharides distinguishes embryonic from adult N-CAM. J.Cell Biol. 103, 2429.

Rutishauser, U. (2008) Polysialic acid in the plasticity of the developing and adult vertebrate nervous system. Nat.Rev.Neurosci. 9, 26.

Rutishauser, U. and Landmesser, L. (1996) Polysialic acid in the vertebrate nervous system: a promoter of plasticity in cell-cell interactions. Trends Neurosci. 19, 422.

Rutishauser, U., Watanabe, M., Silver, J., Troy, F.A. and Vimr, E.R. (1985) Specific alteration of NCAM-mediated cell adhesion by an endoneuraminidase. J.Cell Biol. 101, 1842.

Sáez-Llorens, X. and McCracken, G.H.,Jr. (2003) Bacterial meningitis in children. Lancet 361, 2139.

Saraste, M., Sibbald, P.R. and Wittinghofer, A. (1990) The P-loop--a common motif in ATP- and GTP-binding proteins. Trends Biochem.Sci. 15, 430.

Sarff, L.D., McCracken, G.H., Schiffer, M.S., Glode, M.P., Robbins, J.B., Ørskov, I. and Ørskov, F. (1975) Epidemiology of Escherichia coli K1 in healthy and diseased newborns. Lancet 1, 1099.

Sato, C., Fukuoka, H., Ohta, K., Matsuda, T., Koshino, R., Kobayashi, K., Troy, F.A.,2nd and Kitajima, K. (2000) Frequent occurrence of pre-existing alpha 2-->8-linked disialic and oligosialic acids with chain lengths up to 7 Sia residues in mammalian brain glycoproteins. Prevalence revealed by highly sensitive chemical methods and anti-di-, oligo-, and poly-Sia antibodies specific for defined chain lengths. J.Biol.Chem. 275, 15422.

Sato, C., Inoue, S., Matsuda, T. and Kitajima, K. (1999) Fluorescent-assisted detection of oligosialyl units in glycoconjugates. Anal.Biochem. 266, 102.

Sato, C., Inoue, S., Matsuda, T. and Kitajima, K. (1998) Development of a highly sensitive chemical method for detecting alpha2-->8-linked oligo/polysialic acid residues in glycoproteins blotted on the membrane. Anal.Biochem. 261, 191.

Scheidegger, E.P., Lackie, P.M., Papay, J. and Roth, J. (1994) In vitro and in vivo growth of clonal sublines of human small cell lung carcinoma is modulated by polysialic acid of the neural cell adhesion molecule. Lab.Invest. 70, 95.

Scholl, D., Kieleczawa, J., Kemp, P., Rush, J., Richardson, C.C., Merril, C., Adhya, S. and Molineux, I.J. (2004) Genomic analysis of bacteriophages SP6 and K1-5, an estranged subgroup of the T7 supergroup. J.Mol.Biol. 335, 1151.

Scholl, D., Rogers, S., Adhya, S. and Merril, C.R. (2001) Bacteriophage K1-5 encodes two different tail fiber proteins, allowing it to infect and replicate on both K1 and K5 strains of Escherichia coli. J.Virol. 75, 2509.

Schreiber, S.C., Giehl, K., Kastilan, C., Hasel, C., Mühlenhoff, M., Adler, G., Wedlich, D. and Menke, A. (2008) Polysialylated NCAM represses E-cadherin-mediated cell-cell adhesion in pancreatic tumor cells. Gastroenterology 134, 1555.

Schwarzer, D., Stummeyer, K., Gerardy-Schahn, R. and Mühlenhoff, M. (2007) Characterization of a novel intramolecular chaperone domain conserved in endosialidases and other bacteriophage tail spike and fiber proteins. J.Biol.Chem. 282, 2821.

Schwarzer, D., Stummeyer, K., Haselhorst, T., Freiberger, F., Rode, B., Grove, M., Scheper, T., von Itzstein, M., Mühlenhoff, M. and Gerardy-Schahn, R. (2009) Proteolytic release of the intramolecular chaperone domain confers processivity to endosialidase F. J.Biol.Chem. 284, 9465.

Seidenfaden, R., Krauter, A., Schertzinger, F., Gerardy-Schahn, R. and Hildebrandt, H. (2003) Polysialic acid directs tumor cell growth by controlling heterophilic neural cell adhesion molecule interactions. Mol.Cell.Biol. 23, 5908.

Seki, T. and Arai, Y. (1993) Distribution and possible roles of the highly polysialylated neural cell adhesion molecule (NCAM-H) in the developing and adult central nervous system. Neurosci.Res. 17, 265.

Seki, T. and Arai, Y. (1991) Expression of highly polysialylated NCAM in the neocortex and piriform cortex of the developing and the adult rat. Anat.Embryol.(Berl) 184, 395.

Smith, H.W. and Huggins, M.B. (1982) Successful treatment of experimental Escherichia coli infections in mice using phage: its general superiority over antibiotics. J.Gen.Microbiol. 128, 307.

Stirm, S. and Freund-Mölbert, E. (1971) Escherichia coli capsule bacteriophages. II. Morphology. J.Virol. 8, 330.

Stummeyer, K., Dickmanns, A., Mühlenhoff, M., Gerardy-Schahn, R. and Ficner, R. (2005) Crystal structure of the polysialic acid-degrading endosialidase of bacteriophage K1F. Nat.Struct.Mol.Biol. 12, 90.

Stummeyer, K., Schwarzer, D., Claus, H., Vogel, U., Gerardy-Schahn, R. and Mühlenhoff, M. (2006) Evolution of bacteriophages infecting encapsulated bacteria: lessons from Escherichia coli K1-specific phages. Mol.Microbiol. 60, 1123.

Suzuki, M., Suzuki, M., Nakayama, J., Suzuki, A., Angata, K., Chen, S., Sakai, K., Hagihara, K., Yamaguchi, Y. and Fukuda, M. (2005) Polysialic acid facilitates tumor invasion by glioma cells. Glycobiology 15, 887.

Tanaka, F., Otake, Y., Nakagawa, T., Kawano, Y., Miyahara, R., Li, M., Yanagihara, K., Nakayama, J., Fujimoto, I., Ikenaka, K. and Wada, H. (2000) Expression of Polysialic Acid and STX, a Human Polysialyltransferase, Is Correlated with Tumor Progression in Non-Small Cell Lung Cancer. Cancer Res. 60(11), 3072.

Taylor, C.M. and Roberts, I.S. (2005) Capsular polysaccharides and their role in virulence. Contrib.Microbiol. 12, 55.

Thompson, J.D., Higgins, D.G. and Gibson, T.J. (1994) Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673.

Tsuji, S. (1996) Molecular cloning and functional analysis of sialyltransferases. J.Biochem. 120, 1.

Van Camp, B., Durie, B.G., Spier, C., De Waele, M., Van Riet, I., Vela, E., Frutiger, Y., Richter, L. and Grogan, T.M. (1990) Plasma cells in multiple myeloma express a natural killer cell-associated antigen: CD56 (NKH-1; Leu-19). Blood 76, 377.

Vimr, E.R., McCoy, R.D., Vollger, H.F., Wilkison, N.C. and Troy, F.A. (1984) Use of prokaryotic-derived probes to identify poly(sialic acid) in neonatal neuronal membranes. Proc.Natl.Acad.Sci.U.S.A. 81, 1971.

Vimr, E.R. and Troy, F.A. (1985) Identification of an inducible catabolic system for sialic acids (nan) in Escherichia coli. J.Bacteriol. 164, 845.

Vutskits, L., Djebbara-Hannas, Z., Zhang, H., Paccaud, J.P., Durbec, P., Rougon, G., Muller, D. and Kiss, J.Z. (2001) PSA-NCAM modulates BDNF-dependent survival and differentiation of cortical neurons. Eur.J.Neurosci. 13, 1391.

Walker, J.E., Saraste, M., Runswick, M.J. and Gay, N.J. (1982) Distantly related sequences in the alpha- and beta-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. EMBO J. 1, 945.

Weigele, P.R., Scanlon, E. and King, J. (2003) Homotrimeric, beta-stranded viral adhesins and tail proteins. J.Bacteriol. 185, 4022.

Yang, P., Major, D. and Rutishauser, U. (1994) Role of charge and hydration in effects of polysialic acid on molecular interactions on and between cell membranes. J.Biol.Chem. 269, 23039.

Zhang, H., Vutskits, L., Calaora, V., Durbec, P. and Kiss, J.Z. (2004) A role for the polysialic acid-neural cell adhesion molecule in PDGF-induced chemotaxis of oligodendrocyte precursor cells. J.Cell.Sci. 117, 93.